FINAL REPORT

Ecological Risk Assessment of Munitions Compounds on Coral and Coral Reef Health

SERDP Project ER-2125

January 2014

Cheryl Woodley
NOAA

Craig Downs
Haereticus Environmental Laboratory

SERDP
DOD ▪ EPA ▪ DOE

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JAN 2014		**00-10-2011 to 00-01-2014**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Ecological Risk Assessment of Munitions Compounds on Coral and Coral Reef Health	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **National Oceanic and Atmospheric Administration,Hollings Marine Laboratory,331 Ft Johnson Rd,Charleston,SC,29412**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project was undertaken by the U.S. National Oceanic and Atmospheric Administration (NOAA), National Ocean Service (NOS) National Centers for Coastal Ocean Science (NCCOS) and Haereticus Environmental Laboratory (HEL) to investigate whether munitions compounds (MCs) or their breakdown products impact corals, and to determine the ecological risk they may pose to coral and coral reef health. At the outset of this project, we were asked by the SERDP Scientific Advisory Board (SAB) to modify our originally proposed project by the addition of laboratory toxicity testing. This interim report documents the results for Task 1 of this project, which was to conduct standard laboratory toxicity testing using a coral cell toxicity assay, establishing NOEC and LOEC values and effect-concentration (LC or EC) values.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**142**	

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ORGANIZATION.

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-2014	Technical Report	10-2011-01-2014

4. TITLE AND SUBTITLE

Ecological Risk Assessment of Munitions Compounds on Coral and Coral Reef Health

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Cheryl Woodley
Craig Downs

5d. PROJECT NUMBER

ER-2125

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

National Oceanic and Atmospheric Administration
331 Ft Johnson Rd, Hollings Marine Laboratory
Charleston, SC 29412

8. PERFORMING ORGANIZATION REPORT NUMBER

ER-2125

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

SERDP/ESTCP
4800 Mark Center Drive, Suite 17D08
Alexandria, VA 22350-3605

10. SPONSOR/MONITOR'S ACRONYM(S)

SERDP/ESTCP

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project was undertaken by the U.S. National Oceanic and Atmospheric Administration (NOAA), National Ocean Service (NOS) National Centers for Coastal Ocean Science (NCCOS) and Haereticus Environmental Laboratory (HEL) to investigate whether munitions compounds (MCs) or their breakdown products impact corals, and to determine the ecological risk they may pose to coral and coral reef health. At the outset of this project, we were asked by the SERDP Scientific Advisory Board (SAB) to modify our originally proposed project by the addition of laboratory toxicity testing. This interim report documents the results for Task 1 of this project, which was to conduct standard laboratory toxicity testing using a coral cell toxicity assay, establishing NOEC and LOEC values and effect-concentration (LC or EC) values.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE		143	Stuart Strand
					19b. TELEPHONE NUMBER *(Include area code)* 843-762-8737

Reset

EXECUTIVE SUMMARY

This project was undertaken by the U.S. National Oceanic and Atmospheric Administration (NOAA), National Ocean Service (NOS) National Centers for Coastal Ocean Science (NCCOS) and Haereticus Environmental Laboratory (HEL) to investigate whether munitions compounds (MCs) or their breakdown products impact corals, and to determine the ecological risk they may pose to coral and coral reef health. At the outset of this project, we were asked by the SERDP Scientific Advisory Board (SAB) to modify our originally proposed project by the addition of laboratory toxicity testing. This interim report documents the results for Task 1 of this project, which was to conduct standard laboratory toxicity testing using a coral cell toxicity assay, establishing NOEC and LOEC values and effect-concentration (LC or EC) values. Based on the results from the laboratory experimental efforts, all nine munitions compounds tested were found to have some level of toxicity in one or more of the bioassays conducted. In addition to the activities planned for Task 1 and based on SERDP IPR feedback, supportive tests were conducted with intact coral fragments and cultured coral symbiotic dinoflagellate cells. Tests were also conducted to address the question of whether *in vitro* cell toxicity assays reflect responses of intact coral fragments when they are exposed to MCs.

To understand the environmental relevance of these laboratory findings requires continuing with this project to conduct the ecological risk assessment (ERA) aspect of this proposal, which encompasses Tasks 2, 3, 4 and 5. The ERA is designed to determine which compounds are present in reef environments, their concentrations and to quantify biological effects, validate field data as to type and concentration of specific munitions with intact coral fragments in follow-up laboratory exposures, and to synthesize this information into a risk characterization model that describes the risk exposures to the relevant munitions compounds poses to coral and coral reef health.

Summary of Work to Date

Task 1 objectives were to provide information as to the relative toxicity of key munitions compounds to two coral species and two coral cell types.

- The results of the coral toxicity assay ranks the compounds tested in this system in order of most toxic to least toxic, based on LC_{50} values:

  Picric acid > TNT > 2,4-DNT > 2,6-DNT.

  RDX and HMX median effect concentrations could not be determined because the effect (percent mortality) did not reach the 50% threshold required by PROBIT models to estimate median effect concentrations, even with exposure concentrations in the parts per million (RDX) and parts per thousand range (HMX).

- The photo-enhanced toxicity of TNT was tested with *Pocillopora damicornis* and *Porites divaricata* calicoblast and gastrodermal cells in a 4 h exposure in either light or dark conditions. For *P. damicornis*, the results indicate that both calicoblast and gastrodermal cells were more sensitive to TNT exposure in the light than in the dark, with calicoblast LC_{50} values approximately 100 fold less (16 vs 1582 µg/L) than for cells exposed in darkness). For

P. divaricata, the results indicate that light had little effect on the LC_{50} for the calicoblast cells whether exposed in light or dark conditions, but there was an approximate 20-fold increase in toxicity for gastrodermal cells exposed to TNT in light conditions vs dark. These data are also important in recognizing differences in species sensitivity and the relevance of evaluating multiple species for ecological relevance.

- The difference in species sensitivity to the MC, 2,6-DNT, was tested with three shallow-water coral species, two branching species (*Porites divaricata* and *Pocillopora damicornis*) and one mounding species (*Porites lobata*). Combined within this experimental design were also two cell types of each species, calicoblasts and gastrodermal cells. The results indicate that *P. damicornis* is most sensitive to 2,6-DNT, followed by *P. divaricata*, at an intermediate level and *P. lobata* being the most tolerant to the compound. Gastrodermal cells were found to be more sensitive to the 2,6-DNT than calicoblast cells.

- Seven MCs were tested in static 96 h *Symbiodinium* sp. Clade B cell culture toxicity assays. These included six nitrotoluene compounds: TNT, the parent compound, three dinitrotoluene compounds, including two of its major breakdown products, 2,4-DNT and 2,6-DNT and a minor isomer 2,3-DNT; one nitrotoluene, 4-NT; one aminodinitrotoluene, 2-ADNT; and one nitramine, RDX. Comparisons of relative toxicity among these MCs were evaluated with two physiological endpoints, cell growth and photosynthetic efficiency. Both endpoints gave similar rankings for relative toxicities; however, TNT's relative position in toxicity changed depending on the physiological endpoint. Comparisons of EC_{50} values based on *Symbiodinium* sp. cell growth ranked TNT as the most toxic and 4-NT the least toxic of the nitrotoluenes. RDX exposures had no significant effect on cell growth at any concentration tested up to 15,000 µg/L, which approaches its solubility in seawater (19,770 µg/L at 25°C). Effects on photosynthetic efficiency were similar to those for growth; however, 2,3-DNT appeared to cause the most significant effects on this parameter. RDX had no effect on photosynthetic efficiency.

- TNT was used to test how well the *in vitro cell* toxicity assays reflect responses of intact coral. Realizing coral cells are much more sensitive to toxicants than intact organisms, a correction factor is necessary to translate coral cell mortality into potential mortality of coral fragments. A regression analysis with data from *Porites divaricata* calicoblast and gastrodermal cells (both in light and dark exposure conditions) and intact *P. divaricata* fragments exposed for 96 h to TNT showed that strong positive relationships existed when *in vitro* cell mortalities were regressed against coral fragment necrosis. Each of the regression models performed very well in every case ($p < 0.05$, $r^2 > 0.95$) and each followed the normal distribution (Shapiro-Wilk, $p > 0.05$). Thus, *in vitro* cell mortality may be used to successfully predict coral fragment necrosis.

Table of Contents

List of Tables

List of Figures

List of Acronyms

2,3-DNT	2,3-Dinitrotoluene
2,4-DNT	2,4-Dinitrotoluene
2,6-DNT	2,6-Dinitrotoluene
2-ADNT	2-amino-4,6-dinitrotoluene
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
AOI	Area of interest
ASW	Artificial seawater
BSA	Bovine serum albumin
CAS	Chemical Abstracts Service
CCA	Canonical correlation analysis
CCEHBR	Center for Coastal Environmental Health and Biomolecular Research
DCM	Dichloromethane
DNA	Deoxyribonucleic acid
DNA-AP site	DNA abasic sites (apurinic, apyrimidimic)
DOD	Department of Defense
EC	Effective concentration
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
ERA	Ecological risk assessment
ESA	Endangered Species Act
F_m	Maximum fluorescence signal in dark-adapted chlorophyll fluorescence measurements
F_o	Minimum fluorescence signal in dark-adapted chlorophyll fluorescence measurements
F_t	Fluorescence signal at any time point between minimum and maximum fluorescence signals in chlorophyll fluorescence measurements
F_v	Variable fluorescence in dark-adapted chlorophyll fluorescence measurements
F_v/F_m	Maximum quantum yield in dark-adapted chlorophyll fluorescence measurements
GC-MS	Gas chromatography mass spectrometry
GPS	Global Positioning System
HEL	Haereticus Environmental Laboratory
HML	Hollings Marine Laboratory
HMX	Tetrahexamine tetranitramine (High Melting eXplosive)
HPLC	High performance liquid chromatography
h	Hour(s)
HUFA	Highly unsaturated fatty acid
ICP-MS	Inductively coupled plasma - mass spectrometry
IPR	In progress review
IUPAC	International Union of Pure and Applied Chemistry nomenclature
L	Liter
LC	Lethal concentration

LC_{50}	Median lethal concentration
LC-MS	Liquid chromatography-mass spectrometry
LC-MS-MS	Liquid chromatography tandem mass spectrometry
LED	Light-emitting diode
LOEC	Lowest observed (observable) effect concentration
m	Meter
MC	Munitions compounds
min	Minute(s)
µg	Microgram
µmol	Micromole
mg	Milligram
mL	Milliliter
mtDNA	Mitochondrial deoxyribonucleic acid
NCCOS	National Centers for Coastal Ocean Science
ng	Nanogram
NIST	National Institute of Standard and Technology
NMFS	National Marine Fisheries Service
NOAA	National Oceanic and Atmospheric Administration
NOEC	No observable effect concentration
NOS	National Ocean Service
PAH	Polycyclic aromatic hydrocarbons
PAM	Pulse amplitude modulation
PAR	Photosynthetically active radiation
PCB	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PA	Picric acid or 2,4,6-trinitrophenol
Ppt	Parts per thousand
Ppm	Parts per million
Pptrillion	Parts per trillion
PSII	Photosystem II
PTFE	Polytetrafluoroethylene (Teflon®)
PVPP	Polyvinylpolypyrrolidone
RDX	Cyclotrimethylene trinitramine (Research Department composition X)
s	second
SRM	Standard reference material
SSS	Sigma sea salts
TE	Tris-EDTA buffer
TIE	Toxicity identification evaluation
TNT	2,4,6-Trinitrotoluene
UroI	Uroporphyrin I
USACE	U.S. Army Corps of Engineers
Y(II)	Quantum yield of photochemical energy in PSII
Y(NO)	Quantum yield of non-regulated non-photochemical energy loss in PSII
Y(NPQ)	Quantum yield of regulated non-photochemical energy loss in PSII

Keywords

Coral
Pocillopora damicornis
Porites divaricata
Porites lobata
Calicoblast cells
Gastrodermal cells
Symbiodinium sp.
zooxanthellae
munitions compounds
Ecological Risk Assessment
2,4,6-Trinitrotoluene
2,3-Dinitrotoluene
2,4-Dinitrotoluene
2,6-Dinitrotoluene
4-Nitrotoluene
2-amino-4,6-dinitrotoluene
RDX
HMX
Picric acid

Acknowledgements

This research is being funded by the Strategic Environmental Research and Development Program (SERDP) through a Memorandum of Agreement with the National Oceanic and Atmospheric Administration (NOAA).

Munitions compounds 2,4,6-Trinitrotoluene, RDX and HMX were kindly provided by Dr. Cynthia Price at the U.S. Army Corps of Engineers, Environmental Laboratory, U.S. Army Engineer Research and Development Center, Vicksburg, MS for use in conducting laboratory exposure experiments.

Confirmatory analytical chemical analyses for initial munition compound stability were carried out at NOAA, National Ocean Service, Hollings Marine Laboratory, Charleston, SC. We thank Dr. Ed Wirth and Mr. Jared Ragland of the NOAA NOS NCCOS CCEHBR for their assistance in collecting these data.

We are extremely grateful to Dr. Paul Pennington of NOAA NOS NCCOS CCEHBR for providing the statistical analyses for these data.

We appreciate the time and expertise of Dr. Esther Peters in helpful discussions of the histology of these corals as well as reading the slides for the first 2,3-DNT exposure experiment.

We thank NOAA staff Mr. Thomas Bartlett, Ms. Athena Burnett, Mrs. Lisa May, Ms. Darlene Middleton, Mr. Carl Miller, and Mrs. Laura Webster for their technical assistance on this project in conducting aspects of the design and construction of NOAA's glass and Teflon® system, dosing system for coral fragments, coral fragment exposures, methods development and performing DNA damage and porphyrin assays as well as their assistance in the preparation of this report. We thank Mr. V. Dean Downs of HEL for design and construction of HEL's glass and Teflon® culture and dosing systems.

We also thank Sylvia Galloway, Thomas Bartlett and Lisa May for editorial review of this report and helpful comments.

NOAA Disclaimer
This document does not constitute endorsement of any commercial product or intend to be an opinion beyond scientific or other results obtained by NOAA.

Objectives

SERDP Project ER2125's overall objectives are to evaluate whether munitions compounds (MCs) (e.g., TNT, RDX, Picric Acid, HMX), or their breakdown products impact corals and to determine the ecological risk MCs may pose to coral and coral reef health. The project's Tasks are to:

1. Determine toxicity reference values for targeted munitions compounds by conducting laboratory toxicity testing.
2. Identify specific munition chemicals of concern.
3. Characterize individual and population-level effects of exposure to munitions compounds of concern in the field with laboratory diagnostic assays and in-field assays for determining colony health condition.
4. Determine coral health risks from MC exposure.
5. Identify management screening endpoints and their action values for managing risk to their coral reef health.
6. Establish standard methods for future assessments related to munitions on reefs.
7. Provide cost-effective field methods for DOD monitoring or mitigation activities.

These objectives are being met by conducting laboratory studies to determine toxicity tolerance (effect concentration) and mortality values (lethal concentrations) as a function of specific munitions compound concentrations. Field studies (Phase 2; Tasks 2-3) will determine if toxicity exists at sites with known munitions compounds in proximity to coral reefs using standard Toxicity Identification Evaluation (TIE) protocols and analytical chemistry to identify and quantify the compound(s) and the spatial correlation between concentration of MCs and their proximity to coral colonies. Along with the TIE (effects characterization) and chemical analyses (exposure characterization), tissue and cellular physiological profiles from reference and impacted sites will be used to characterize biological effects at MC-containing sites. Once environmentally relevant types and levels of MC are determined, whole-animal (intact coral fragments) laboratory testing will be used to determine whole animal toxicity reference values (Task 4). The project will culminate with the synthesis of this information into a risk characterization model that a) describes the risk to coral health of exposures to munitions compounds, b) identifies screening and action values, and c) identifies uncertainty factors in the risk model (Task 5).

This interim report addresses Objectives 1 and 2 and is tied to the Go/No-go decision point of this project.

Technical Approach

Coral Culture Systems

HEL's Coral Culture System

The coral species *Pocillopora damicornis* and *Porites divaricata* coral species were grown in a glass/Teflon® multi-tank system at Haereticus Environmental Laboratory (HEL) (Fig. 1). Each coral species was cultured in separate and independent tank systems under custom LED lighting that had a wavelength range from 400 nm to 710 nm, with a maximum planar incidence exposure of 248-295 µmoles of photons $m^{-2}s^{-1}$. The system's light cycle was 8 hours (h) of light and 16 h of darkness. Teflon®-lined Iwaki pumps (Model MD-55LFZ-115) were used to circulate 35 parts per thousand (ppt) artificial seawater (Instant Ocean, Blacksburg, VA) at a flow velocity of ~24 liters per min (6.4 gal/min). In addition to day-light lighting, a proprietary LED "moonlight" was activated based on a lunar cycle (28 days) that ranged from darkness to a maximum of 13 µmoles of photons $m^{-2}s^{-1}$ planar incidence. Corals were fed every 72 h with a custom coral feed formulation that was tested for heavy metal contamination. Corals experienced these culture conditions at least two months before cell isolation and toxicology exposures.

Figure 1. HELGlass-Teflon Coral Culture System. The experimental system is fabricated with all glass and Teflon® parts, the lighting system, developed by HEL, uses custom made LED fixtures designed with detailed wavelengths of light and spatial array for optimal coral growth and individualized for each species. *Photo by Craig Downs.*

NOAA's Coral Culture System

Pocillopora damicornis coral was cultured in a similar glass/Teflon® system at NOAA (Fig. 2). Since the proprietary custom-made LED lighting used by HEL was not available, lighting for this system used a 48" 324 watt Sunlight Supply Tek Light T5 HO fluorescent light fixture fitted with three 54 watt AquaSun 10,000 K T5 HO fluorescent bulbs (UV Lighting International, Brook Park, OH) and three 54 watt ATI Blue Plus T5 HO fluorescent bulbs in an alternating configuration to approximate HEL's custom LEDs and

Figure 2. NOAA Glass-Teflon® Coral Culture System. The deeper tank design allows propagation of larger parent colonies for producing clonal fragments for exposure experiments. Shown above are three parent colonies of *Pocillopora damicornis* and fragments on Teflon® pegs and stands that were custom designed and fabricated by in-house staff. *Photo by Carl Miller.*

adjusted to 150±20 µmoles $m^{-2}s^{-1}$ of photosynthetically active radiation (PAR). For culturing colonies as well as fragments, the NOAA tank was designed deeper (24" wide x 60" long x 16" deep), with a tank volume of approximately 125 gallons (470 liters). A Teflon®-lined Iwaki pump (Model: MD-55LFZ-115) circulates 35 ppt Reef Crystals artificial seawater (Instant Ocean, Blacksburg, VA) at a rate of approximately 40 L per min (10.5 gal/min). Corals were fed three times weekly with approximately 2.5 gm of a custom formulated food of particle sizes ranging from 50-1700 microns with high protein and highly unsaturated fatty acid (HUFA) content.

Munitions Compounds Tested

Stock solutions of RDX, HMX and TNT in methanol or acetone were provided by USACE Environmental Laboratory, Vicksburg, MS. Picric acid was obtained from Electron Microscopy Sciences (Hatfield, PA) (cat # RT19550). Remaining compounds were purchased from ChemService (West Chester, PA): TNT (Cat# N-10659), 2,3-DNT (Cat# NG-16399), 2,4-DNT (Cat# N-10643), 2,6-DNT (Cat# NG-10697), 2-ADNT (Cat# N-10268), 4-NT (N-12787).

Table 1. List of munitions compounds and breakdown products tested.

Common name	IUPAC name	CAS #	Specimen Tested
TNT, Trinitrotoluene	2-methyl-1,3,5-trinitrobenzene	118-96-7	Coral cells, Pdiv frags, Dino
2,3-Dinitrotoluene, 2,3-DNT	1-methyl-2,3-dinitrobenzene	602-01-7	Pdam frags, Dino
2,4-Dinitrotoluene, 2,4-DNT	1-methyl-2,4-dinitrobenzene	121-14-2	Coral cells, Dino
2,6-Dinitrotoluene, 2,6-DNT	1-methyl-2,4-dinitrobenzene	606-20-2	Coral cells, Dino
4-Nitrotoluene, 4-NT	1-methyl-4-nitrobenzene	99-99-0	Dino
2-Amino-4,6-Dinitrotoluene, 2-ADNT	2-methyl-3,5-dinitroaniline	35572-78-2	Dino
RDX, cyclonite, hexogen, T4	1,3,5-trinitroperhydro-1,3,5-triazine	121-82-4	Coral cells, Pdam frags, Dino
HMX, octogen	1,3,5,7-tetranitro-1,3,5,7-tetrazocane	2691-41-0	Coral cells
Picric Acid	2,4,6-trinitrophenol	88-89-1	Coral cells

Pdam frags= *Pocillopora damicornis* coral fragments, Pdiv frags= *Porites divaricata* coral fragments, Dino= symbiotic dinoflagellate, *Symbiodinium* sp. Clade B isolated from *Pocillopora damicornis*.

Coral Cell Toxicity Assay

Primary cell cultures were prepared by disaggregation using mechanical and enzymatic separation of tissue from the coral skeleton (Fig 3). Tissue/skeleton was crushed using a wrenching vise-grip (Fig. 3A). Lysozyme (100 µg) and amylase (100 µg) were added to 100 mL of artificial seawater (ASW; Sigma Sea Salts (SSS), Cat#S9883) and incubated with the coral tissue. Cells were incubated for 10 min in this solution to remove mucus. Dispase (100 µg) was then added to the tissue/skeleton slurry and incubated for 15 min (Fig. 3B). The tissue slurry was mixed on a rocker and gently agitated with a small whisk (not shown) to assist in cell disaggregation. Once tissue disaggregation occurred, the cell slurry was then subjected to two differential centrifugations using a swinging bucket rotor at 50 *x g* for 5 min. The cell slurry was then layered onto a Percoll® step-gradient (as in Downs et al. 2010; 2013), and centrifuged at approximately 200 *x g* for 4 min (not shown). Different fractions of non-zooxanthella (term used for dinoflagellate symbiont, *Symbiodinium* sp.) containing cells (Figs. 3C,D) were collected, stained with Mitotracker® Red (Life Technologies, Grand Island, NY), washed and then assayed using a fluorescent plate reader. Calicoblasts were identified by having the highest intensity of red fluorescence; the largest number of mitochondria per cell (Davy et al. 2012). Cells were cultured in a modified tissue culture media for 48 h (Downs et al. 2010; 2013). Coral cell-culture media (pH 8.2) consisting of 1 mL RPMI-1640, 100x vitamin solution to 99 mL of SSS-ASW (36 ppt salinity), 50 mM Hepes/KOH (pH 8.2), 1 mM calcium chloride, 1 mM sodium pyruvate, 0.075 g/L D-glucose, 0.3 g/L galactose, 0.25 g/L sodium DL-lactate, 0.25 mM ascorbate, 0.05 g/L α-lipoic acid, 0.5 mM proline, 0.5 mM cysteine, 0.5 mM methionine, 0.01 mM hydroxycobalamin, 0.001 mM sodium folate, 1 g/L bovine albumin (V), 0.05 g/L succinate, and 0.25 g/L L-glutamine. Cells were cultured for 48 h in the dark with media changes every 8 h. Before use for toxicity studies, cells were centrifuged at 70 *x g* for 3 min, the pellet resuspended in 5 mL of tissue culture media followed by a cell count. The cell suspension was then layered on a 20% Percoll®/Ficoll cushion and centrifuged in a swinging-bucket rotor at 60 *x g* for 3 min. Cells were collected nad the Percoll®/Ficoll was removed by centrifuging cells at 70 *x g* for 3 min, resuspending the pellet in culture media and repeating twice. The resulting pellet was collected and

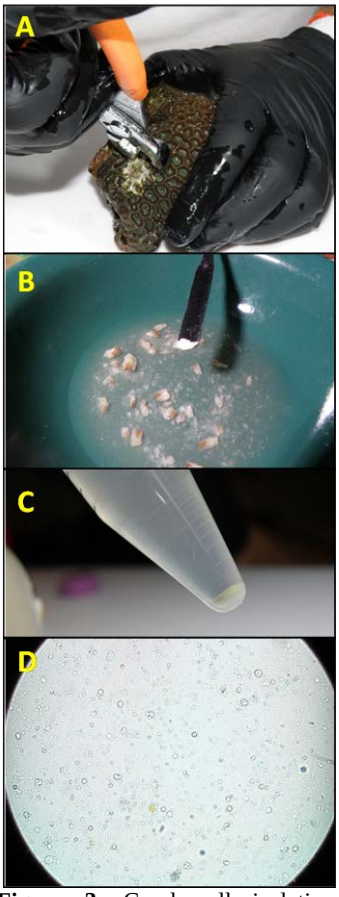


Figure 3. Coral cell isolation procedure. (A) Mechanical separation of coral tissue/skeleton. (B) Enzymatic digestion of tissue with dispase neutral protease and glycosidases. (C) Calicoblast cell pellet after density gradient separation. (D) Photomicrograph of non-gastrodermal coral cell fraction.

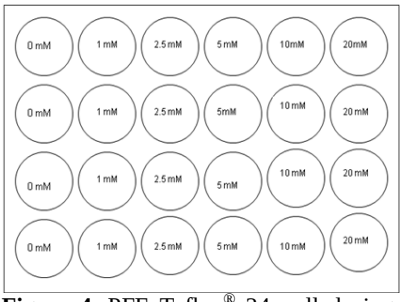


Figure 4. PFE Teflon® 24-well dosing plate scheme.

resuspended in an appropriate volume of tissue culture media. An aliquot of the resulting cell suspension was counted with the vitality stain, amido black or trypan blue, to determine the live cell count prior to initiating the toxicity testing. Stain was prepared by adding 0.5 mg to 10 mL of culture media. The mixture was stirred using a Teflon® stir-bar for 30 min at 26°C and then filtered using Nylon 5 micron sieve filter paper. Equal volumes (500 µL) of stain solution and coral cell suspension were mixed in a 1.8 mL microcentrifuge tube and incubated at room temperature at least 5 min before microscope examination. Approximately $1.0x10^6$ cells per replicate were distributed into treatment solutions in 24-well custom-made PTFE plates (Fig. 4) and incubated for 4 h in the light or dark conditions at 26°C. Light treatments were conducted at 295 µmoles $m^{-2}s^{-1}$ PAR using the same custom LED light fixtures as for the parent colony culture. Cells were again counted to determine cell mortality after exposure to the compound.

***Symbiodinium* sp. Toxicity Exposures and Cell Viability Assays**

Symbiodinium sp. are single-celled dinoflagellate algae that are most often found in nature in symbiotic relationships, but which can occur as free-living cells. Many cnidarian and coral species host these dinoflagellate algae as an endosymbiont that resides intracellularly within coral gastrodermal cells. The NOAA laboratory has isolated *Symbiodinium* sp. from several coral species and established non-axenic *in vitro* cultures. The cultures are grown in L-1 media (35.0 ± 0.5ppt) under LED lights (white/blue/green) (Solaris) with an intensity of approximately 100 µmoles m^{-2} s^{-1} (10h:14h, light:dark) at 26 ± 0.5°C. We selected the algal culture isolated from *Pocillopora damicornis* for toxicity testing. These dinoflagellates have been genotyped as Clade B based on DNA sequence of the small and large subunit ribosomal gene and the internal transcribed spacer regions. Clade B S*ymbiodinium* cells from *P. damicornis* have been shown to have decreasing growth rates at temperatures above 27°C (Kinzie et al. 2001) but generally are found to tolerate a wide range of light and temperature conditions allowing survival and growth (Karako-Lampert et al. 2005). When associated with coral, these dinoflagellates are also commonly referred to as zooxanthellae. These terms are used interchangeably in this report.

Symbiodinium testing protocols were developed with guidance from EPA's *Ecological Effects Test Guidelines OPPTS 850.5400 Algal Toxicity, Tiers I and II* (EPA 712-C-96-164) and adapted from ASTM, 1996, Vol. 11.05, pp. 29-33. The protocol used here measures the chronic toxicity of the selected chemical during a four-day static exposure without replenishment. Munition compound working stocks were prepared by diluting the concentrated stock solution in acetone with fresh L-1 medium (34.5 ppt) (Cat# MKL150L; National Center for Marine Algae and Microbiota, Ease Boothbay, ME) and adding the appropriate volume of 100% acetone so that when combined with the *Symbiodinium* stock culture, the final concentration of acetone was 0.05% for all treatments. Working stock solutions of MCs were prepared in EPA-cleaned 40 mL Teflon® vials, covered with foil, and allowed to mix for at least 30-45 min (to overnight for some compounds) at 26°C using an EPA-cleaned Teflon® stir bar. One milliliter of the working stock solution was transferred to a clean, foil-wrapped glass tube and archived at 4°C for chemical analysis. Exposure treatment solutions were prepared by combining the appropriate volumes of the munition compound working stock, *Symbiodinium* stock culture ($2.0x10^5$ cells/mL final), and 0.2% acetone in L-1 medium (0.05% final) in an EPA-cleaned 40 mL Teflon® vial. Treatments were randomly designated a letter, and 5 mL of each treatment solution were aliquoted in

triplicate into randomly numbered 5 mL EPA-cleaned Teflon® vials for exposure at 26°C and a light intensity of 85-105 µmoles $m^{-2}s^{-1}$ PAR (Solaris fixture equipped with white/blue/green LED lights) and a 10 h:14 h (light:dark) regime. Each day the dosed *Symbiodinium* cultures were carefully mixed to prevent clumping of cells, and temperature and salinity were measured. At the 48 h and 96 h time points, cells from each treatment were counted using a C-Chip Neubauer Improved disposable hemacytometer (Incyto, Korea). *Symbiodinium* cell counts were determined from all eighteen of the larger grid squares within the two chambers. Concurrently, 500 to 1000 µL were transferred from each vial to a 1.5 mL Eppendorf tube to be used for PAM fluorometric analysis.

Pulse-Amplitude Modulated (PAM) Fluorometry

Table 2. Common parameters measured by PAM fluorometry and their definitions.

Fv/Fm	**Maximum quantum yield**
	A measure of available reaction centers. Greater stress, fewer reaction centers are available and ratio is lowered. Proportional to quantum yield of photosynthesis.
Y(II)	**Quantum yield of photochemical energy conversion in PSII**
	Corresponds to the fraction of energy photochemically converted, i.e., using light to produce ATP & NADPH.
Y(NPQ)	**Quantum yield of regulated non-photochemical energy loss in PSII**
	Corresponds to the fraction of energy dissipated in the form of heat through photoprotective mechanisms (heat dissipation, xanthophyll cycling, photorespiration). High values are indicative of high photoprotective capacity.
Y(NO)	**Quantum yield of non-regulated non-photochemical energy loss in PSII**
	Reflects the fraction of energy passively dissipated in the form of heat and fluorescence, mainly due to closed PSII reaction centers (chlorophyll bleaching, protein denaturation, etc). High values reflect inability to protect against damage from excess illumination.
Optimum conditions: high maximal yields of Y(II) and maximal ratio of Y(NPQ)/Y(NO). At saturating pulses high Y(NO) and low Y(NPQ) are suboptimal capacity for photoprotection and will lead to photodamage. High Y(NO) after dark-adaption is indicative of such damage.	

Selection Criteria for *Symbiodinium* Clade B Stock Cultures Used in Toxicity Testing

The selection of healthy cultures of the symbiotic dinoflagellate, *Symbiodinium* sp. Clade B was critical to the toxicological testing. To be considered healthy, the cultures should exhibit characteristic growth curve doubling times (approx. 60 h for Clade B isolate). In addition, we also included criteria that evaluated the culture's photosynthetic efficiency as being optimal for that isolate and the particular culture. Chlorophyll fluorescence measurements using an Imaging

PAM fluorometer (Ralph et al. 2005) was used for these determinations (Table 2 provides a guide to terms used in these analyses). To determine the photosynthetic efficiency of a culture, the initial concentration of the stock culture was determined using a Bright-Line® Improved Neubauer hemacytometer. A subsample of the stock culture was then diluted to $1.43x10^5$ cells/mL with L-1 media and 350 µL of the diluted culture was added in triplicate to a clean Nunc® 96-well, flat-bottom, white MaxiSorp® microtiter plate (Thermo Scientific™, Waltham, MA) for a final concentration of $5x10^4$ cells/well. The plated cells were allowed to sit under the laboratory ambient light for 5 min and were then dark-adapted in the closed MAXI-Imaging PAM chlorophyll fluorometer (Waltz, Effeltrich, Germany) for another 5 min without the measuring light being activated. After dark-adaptation, the measuring light was activated for 15 sec to allow the Ft signal in the filled wells to stabilize. Once stabilized, a Kautsky curve analysis was performed using the settings controlled by the accompanying ImagingWin v2.40b software (measuring light intensity=2, actinic light intensity=5, saturation pulse intensity=10, delay=40 sec, clock=40 sec, run time=755 sec, F_m correction factor=1.055). A Cinegel N.6 neutral density filter (Rosco Laboratories, Stamford, CT) was used to attenuate the intensity of the PAM fluorometer's LED light source such that the fluorescent signal of the *Symbiodinium* did not exceed the detectable limit of the fluorometer. *Symbiodinium* cultures yielding a maximum quantum yield (Fv/Fm) measurement of 0.500 to 0.550 were considered healthy and suitable for use in the dosing experiment (Fig. 5).

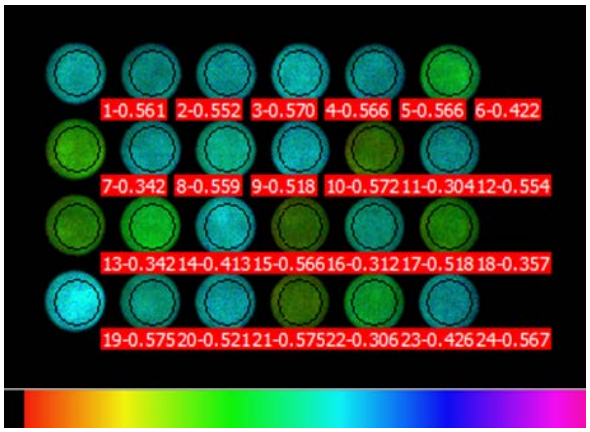


Figure 5. Image of dosed *Symbiodinium* cultures displayed by the Imaging-PAM. The image is displaying the maximum quantum yield of cultures dosed with 2,3-dinitrotoluene after 96 h. The false color scale helps demonstrate which cultures have been negatively affected by the compound. Impaired photosynthetic efficiency is represented by greener wells while less affected cultures remain bluer.

Analysis of Dosed Experimental *Symbiodinium* sp. Cultures

During daily cell counts of the dosed *Symbiodinium* cultures, a 0.5 to 1.0 mL aliquot was removed from the dosing vessel to a 1.5 mL Eppendorf tube. Based on the subsequent cell counts for each sample, the *Symbiodinium* aliquots were diluted to $1.43x10^5$ cells/mL with L-1 media and 350 µL of the diluted culture was added in triplicate to a clean Nunc® 96-well, flat-bottom, white MaxiSorp® microtiter plate for a final concentration of $5x10^4$ cells/well. Triplicates were randomized within the central 24 wells of the microtiter plate. A Kautsky curve analysis was performed as described above for the initial *Symbiodinium* sp. stock cultures (Fig. 5). From the Kautsky curve data, values for the parameters Y(II), Y(NO), and Y(NPQ) were averaged over four time-points when measurements stabilized (Table 2), and these parameters where compared for all treatments.

Analysis of Dosed Coral Fragments

PAM analysis was performed on the same randomly selected, predetermined fragments throughout the entire course of the dosing experiment. The position of these fragments within their dosing beakers and their orientation under the PAM fluorometer were designated by matching notches in the Teflon® stands and bases, respectively. At the time of analysis (T=0, 14, 48, and 96 h), the four replicates for each treatment were placed in a clean 150 mm x 75 mm Pyrex crystallization dish containing 200 mL of new artificial seawater mixed the night before. A new dish was used for each treatment. The orientation of the replicates was consistently maintained by positioning the fragments horizontally in a clean Teflon® stand with their notched side facing upwards.

PAM analysis for each treatment at each time point followed the same routine. Fragments were orientated in the Teflon® stand under the fluorometer camera using infrared light supplied by the instrument. The measuring light (intensity=1) was activated and a circular area of interest (AOI) was placed near the middle of each replicate for reference purposes. The measuring light was deactivated and the fragments dark-adapted inside the closed fluorometer for 5 min. The measuring light was activated again and the Ft signal was allowed to stabilize for 15 sec. A saturation pulse (intensity=10) was initiated in order to determine the dark-adapted maximum photosynthetic quantum yield (Fv/Fm). The fragments were then returned to their respective beakers containing replenished treatments prepared in artificial seawater.

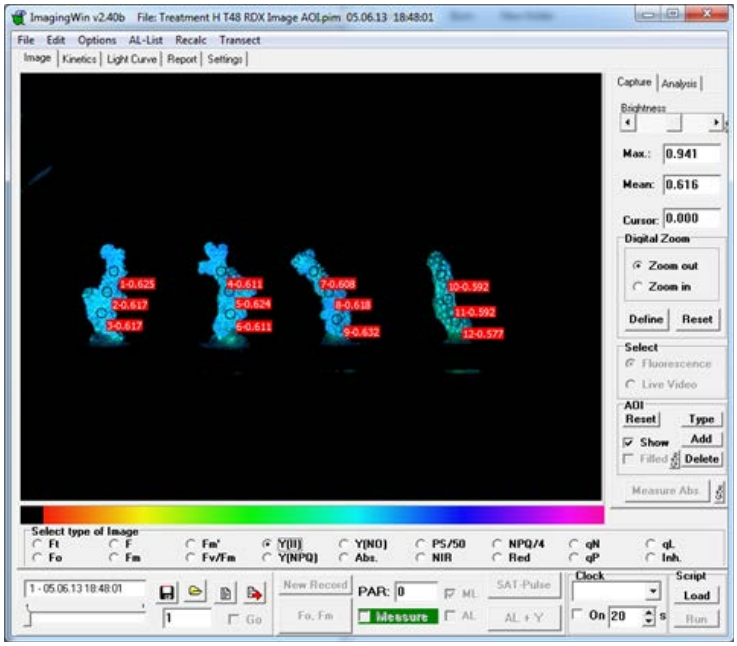


Figure 6. Example of maximum photosynthetic quantum yield (Fv/Fm) analysis in RDX dosed *Pocillopora damicornis*. Using a Waltz Imaging-PAM fluorometer equipped with ImagingWin v2.40b software, the overall Fv/Fm (red text boxes) for each replicate is averaged from three separate areas of interest (black circles on coral fragment images) placed along the coral fragment's length.

Comparisons between treatments and time points were made by placing three new circular AOIs on each replicate. These AOIs were positioned so as to provide Fv/Fm values over the length of the coral fragment while avoiding the *Symbiodinium* deplete areas of the apical growth tip and fragment base (Fig. 6). The position of the AOIs on each fragment was kept constant throughout the time points by pictorial comparison. The three AOIs on each replicate were then averaged to provide a single Fv/Fm value for that replicate.

Application Notes on Coral Fragment PAM Analysis

We have found that, at least within our coral holdings and those of the Co-PI, some species do not respond to PAM fluorometric analysis as expected when compared to *Pocillopora damicornis*. The dissimilarities were most notable in different *Porites* species and some Acroporids. Particularly amongst the *Porites* examples, these fragments were characterized by very high initial fluorescence (F or Fo), low maximum fluorescence (Fm or Fm') after a saturation pulse, and consequently low quantum yields (Y(II) or Fv/Fm) (Fig. 7). These

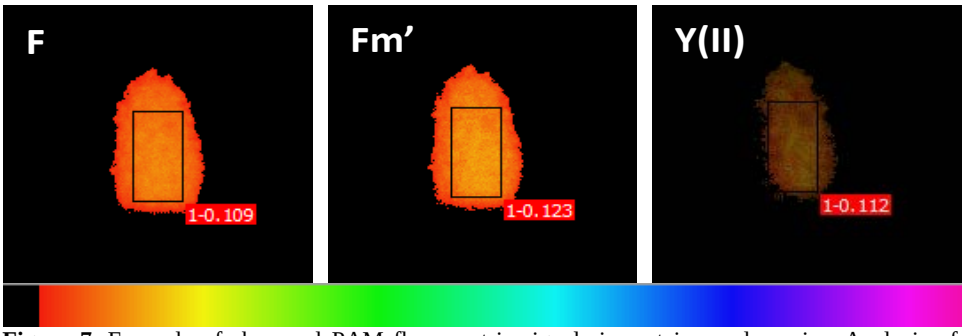


Figure 7. Example of abnormal PAM fluorometric signals in certain coral species. Analysis of *Porites divaricata* yielded a comparatively high initial fluorescence (F). In order to achieve an acceptable initial fluorescence signal (F=0.100-0.200), the measuring light had to be decreased to the lowest value (1), and a ND.6 neutral density filter was used to attenuate the LED light source even more. A saturation pulse produced a low maximum fluorescence (Fm') and, consequently, a relatively low quantum yield (Y(II)); whereas Fm' and Y(II) measurements for *Pocillopora damicornis* are commonly two- and six-fold greater, respectively.

parameters were not significantly affected (i.e., improved) by any length of dark-adaptation (from minutes to hours). Moreover, these characteristics appeared to be genera/species specific; other coral genera under the same culture conditions (i.e., same tank, water chemistry, light regime) did not perform in the same manner. However, *Acropora*/*Porites* species cultured in different tanks under different light sources behaved similarly (Fig. 8). Thus we learned that classical PAM analysis, as it has been applied in the literature, is not feasible for certain coral species. Until the underlying cause of these anomalies can be determined, caution should be used when selecting coral species for studies requiring the use of the PAM.

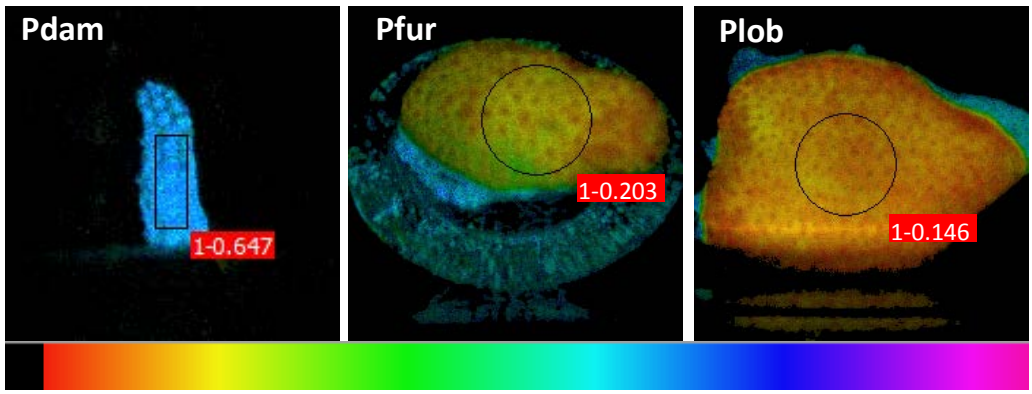


Figure 8. Photosynthetic quantum yields (Y(II)), highlighted in red) of *Pocillopora damicornis* (Pdam), *Porites furcata* (Pfur), and *Porites lobata* (Plob). Pdam and Pfur were both cultured under the same conditions as *P. divaricata* (above), while Plob was cultured in a separate tank under a different light regime.

Coral Fragment Exposures

Porites divaricata were fragmented and cultured at HEL for at least 30 days prior to an experiment. The experimental design involved replicated treatments (n=9) with treatment concentrations of 5, 25, 100, 500, 2,500, 12,500, 25,000, and 50,000 µg/L 2,4,6-trinitrotoluene (TNT), plus controls (Fig. 9). Fragments were removed from the exposure vessels at 16 h, 24 h, and 96 h time points. Three replicates were flash frozen in liquid nitrogen for porphyrin and DNA AP site assays, three replicates were fixed in seawater adjusted Z-fix for light microscopy for tissue-level pathologies, and three replicates were fixed in glutaraldehyde-paraformaldehyde for electron microscopy to examine sub-cellular pathologies. Exposure treatment solutions were replenished every 8 h over the course of the 96 h. Exposures began in the dark for 16 h, with fragments preserved before the beginning of the light cycle (295 µmoles m^{-2} sec^{-1} PAR). A second set of samples was preserved at the 24 h time point representing an exposure for 16 h in the dark followed by 8 h of light. The 96 h samples experienced three dark-light (16:8 h) cycles. Each of the frozen samples were hand ground in liquid nitrogen-cooled mortar and pestle into a fine powder consistency for DNA AP site and total porphyrin quantification assays. Fixed tissue samples were processed for light microscopy.

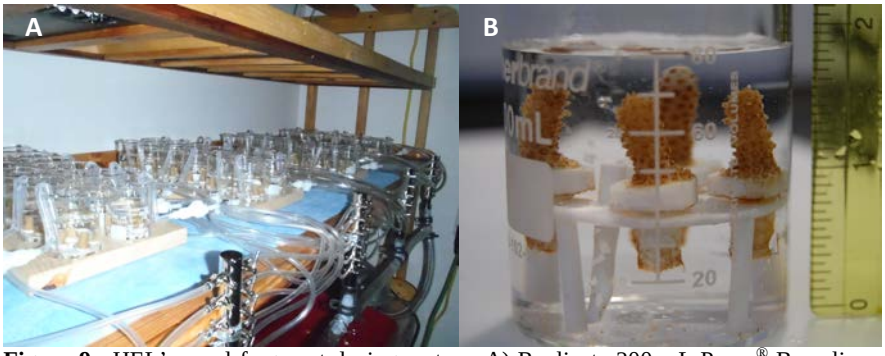


Figure 9. HEL's coral fragment dosing system. A) Replicate 200 mL Pyrex® Berzelius glass beakers were fitted with Teflon® airlines, controlled with a stainless steel manifold with individual control valves. Lighting was from custom designed and built LED lights. B) Example of an individual dosing chamber fitted with custom made Teflon® stand and pegs to support coral fragments. *Photos by Craig Downs.*

Pocillopora damicornis fragments were exposed to munition compounds at NOAA in a time-course design for 96 h. Exposure temperature was maintained at 26°C in an environmentally controlled room. Lighting was provided by a 48" 324 watt Sunlight Supply Tek Light T5 HO Fluorescent light fixture fitted with three 54 watt AquaSun 10,000 K T5 HO fluorescent bulbs (UV Lighting International) and three 54 watt ATI Blue Plus T5 HO fluorescent bulbs in an alternating configuration. The light fixture was centered over the experimental dosing setup and adjusted to supply 150 ± 20 µmoles m^{-2} s^{-1} of PAR (except fragments dosed with RDX in which case the light intensity was 85 µmoles $m^{-2}s^{-1}$). Treatments were conducted in 300 mL Berzelius glass beakers containing 200 mL of treatment solution. Coral fragments were held in position in the water column with custom-made Teflon® pegs and Teflon® supports. Each treatment vessel was individually aerated with a custom-made manifold and Teflon® tubing, and airflow adjusted with individual gang valves (Fig. 10).

The 2,3-DNT experiments were designed with six replicates for each of five treatments with four fragments in each treatment replicate to allow for repeated sampling. RDX dosing was designed with four replicates for each of seven treatments and four fragments in each treatment replicate for time point sampling. Treatment dosages were based on the ranges found toxic to arthropods and fishes as well as compound solubility and preliminary tests with cultures of dinoflagellate algae. Treatments included a carrier (acetone) control in SSS-ASW (Sigma Aldrich) adjusted to 35 ppt, with all solutions replenished every 12 h. Fragments were removed from the exposure vessels at 0 h, 14 h (dark), 24 h (14 h dark: 8h light), 48 h and 96 h time points. Each day the fragments were photographed and scored visually for physiological condition. At 0 h three replicate fragments were flash frozen (as baseline from culture conditions) and one fragment fixed for light microscopy. At the 14 h dark, 24 h and 48 h sampling points, one fragment was frozen from each replicate. At 96 h a final fragment from each replicate was frozen and another fixed for histology. Alternatively, during RDX dosing, at each time point one fragment from each replicate was flash frozen in liquid nitrogen for cellular physiological assays while the same, designated fragment was analyzed by PAM fluorometry (using a repeated measures design) and returned to its dosing container. After the 96 h PAM reading, the designated fragment was placed in Z-fix for histological analysis.

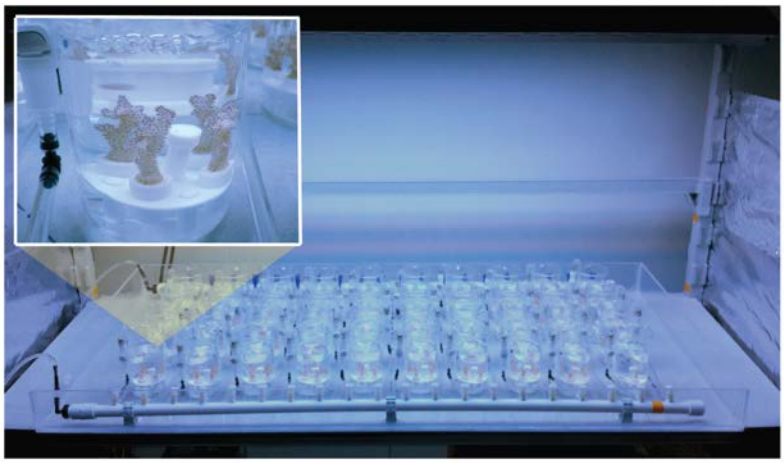


Figure 10. NOAA's coral fragment dosing system. Replicate 300 mL Pyrex® Berzelius glass beakers are fitted with Teflon® airlines, controlled with individual valves to control airflow in each beaker. Lighting is from 48" fluorescent lights. Example of an individual dosing chamber fitted with custom made Teflon® stand and pegs to support coral fragments (Inset photo). *Photos by Thomas Bartlett.*

Organic Extraction of Porphyrin from Coral Tissue

Porphyrins are macrocyclic compounds of the heme biosynthetic pathway and are ubiquitous in nature, forming the basic structure of hemoproteins that are found in most major metabolic pathways that include detoxification systems, respiration, and oxidative metabolism (e.g., chlorophyll, hemoglobin, cytochromes and catalase) (Fig. 11). The porphyrin (heme) biosynthetic pathway is also known to be susceptible to various anthropogenic toxicants (e.g., pesticides, PCBs, PAHs, heavy metals). These can affect different enzymatic steps in the pathway and depending on which step is affected, can lead to either an accumulation or depression in levels of porphyrin precursor species. Many of these intermediate porphyrin species can readily absorb light and create secondary damage from their generation of reactive oxygen species.

Porphyrin intermediates exhibit a characteristic absorption at 400-420 nm with fluorescent emission spectra between 550 and 650 nm. These excitation emission parameters form the basis for a sensitive and specific diagnostic marker. This human diagnostic has been adapted for use in coral tissue and is an indicator for generalized metabolic disruption (Avadanei et al. 2012).

This modified method, based on an organic extraction of the tissue, was developed and optimized during the first phase of this project to minimize the amount of tissue needed to measure porphyrin levels in coral and for potential use in coral cell bioassays. The modification is improved over the originally proposed method involving aqueous detergent extraction of total soluble proteins by reducing the amount of tissue require to quantify this important endpoint from approx. 100-200 mg to 25-50 mg of coral tissue/skeleton, depending on the species of coral and reducing the time required to perform the assay. It was determined that a minimum of $5x10^5$ to $1x10^6$ cells would be required for cell-based assays.

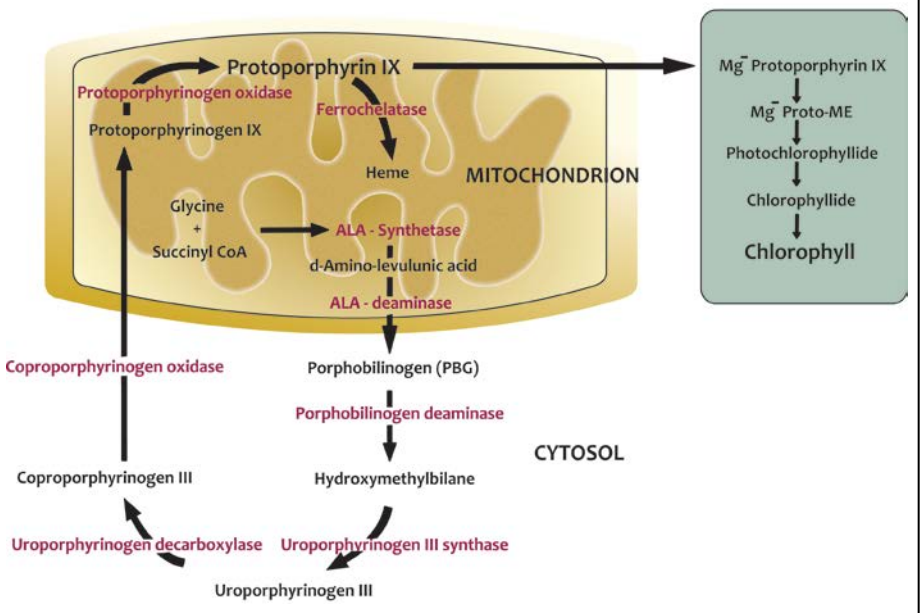


Figure 11. Diagram of the Heme Biosynthetic Pathway. *Artwork by Athena Burnett.*

Extraction of coral tissue

Approximately 50 µL of frozen ground coral tissue was transferred to a chilled microcentrifuge tube using a chilled stainless steel spatula. Samples were kept frozen at -80°C until extracted. At the time of extraction, samples were warmed to room temperature, and 500 µL of extraction buffer (25% acetonitrile in 50 mM phosphate buffer, pH 8.0) was added to each and quickly vortexed to mix. The coral tissue was disrupted (without addition of silica microbeads) using a Mini-Beadbeater-8 (BioSpec Products, Inc., Bartlesville, OK) kept chilled at 4°C in a walk-in refrigerator. The tissue was mixed for two cycles of one minute each at maximum speed with a one minute interval between each cycle. The tissue homogenate was then incubated at room temperature for 15 min, vortexing every 5 min, to increase the interaction between the coral tissue and the extraction buffer. After the incubation, coral tissue homogenates were centrifuged at 20,817 *x g* for 15 min and the supernatant transferred to a new amber microcentrifuge tube. All subsequent assays were performed under attenuated light.

Protein Concentration Determination

A five-point protein standard was prepared from a 200 mg/mL protein standard stock (bovine serum albumin (BSA); Sigma, St. Louis, MO). A 10 mg/mL BSA working stock was first made by diluting the BSA protein standard 1:20 in phosphate buffer. The working stock was then diluted to 800 µg/mL in extraction buffer. Subsequently, two-fold serial dilutions were prepared by diluting the BSA working stock 1:1 in extraction buffer (standard range=50-800 µg/mL BSA). Extraction buffer without BSA was included as a blank.

In a new microcentrifuge tube, 80 µL of coral tissue extract or BSA standard was added to 415 µL of phosphate buffer. After vortexing the sample, 55 µL of 1 mg/mL fluorescamine (Acros Organics, New Jersey) in acetonitrile was added and quickly vortexed to mix. After fluorescamine was added to each sample, 150 µL of the mixture was aliquoted in triplicate to a 96-well Nunc® optical bottom black plate (Nalge Nunc® International, Rochester, NY). Approximately 15-20 min after the last sample was mixed and all samples were plated, fluorescence was measured using a Bio-Tek® Synergy™ HT microplate reader (Bio-Tek® Instruments, Inc.) fitted with 400 ± 15 nm excitation and 460 ± 20 nm emission filters. A standard curve was generated and sample total soluble protein concentrations were calculated using the associated Bio-Tek® KC4 microplate data analysis software. Using the determined concentrations, samples were adjusted to 10 µg/mL total soluble protein by diluting an aliquot of the original extraction with extraction buffer in a new amber microcentrifuge tube.

Total Porphyrin Quantification

A five-point standard was prepared from a 0.5 µg/mL Uroporphyrin I (UroI) fluorescent standard stock in 1 N HCl (Frontier Scientific, Inc., Logan, UT). UroI served as a proxy for measuring total porphyrin concentration in the extracted coral tissue. A 0.5 µM UroI working stock was first made by diluting the 0.5 µg/mL stock with 1 N HCl. An initial 1.0 pmol UroI standard was prepared by diluting the UroI working stock to a final concentration of 1.0 pmol/150 µL (6.67 pmol/mL) in extraction buffer containing 10 µg/mL BSA (final concentration; obtained from the 800 µg/mL BSA standard prepared earlier). Subsequently, two-fold serial dilutions were prepared by diluting the 1.0 pmol UroI standard 1:1 in extraction buffer containing 10 µg/mL BSA (standard range= 0.0625-0.50 pmol). Extraction buffer with 10 µg/mL BSA was included as a blank.

To a new 96-well Nunc® optical bottom black plate, 150 µL of diluted extract or UroI standard was aliquoted in triplicate and then 30 µL of 6 N HCl was added to each well. The plate was immediately shaken two times for 20 s each at low intensity using the Bio-Tek® microplate reader and incubated at room temperature in the dark for 10 min. After incubating, fluorescence was measured using the Bio-Tek® microplate reader fitted with 400 ± 15 nm excitation and 600 ± 20 nm emission filters. A standard curve was generated and sample total porphyrin concentrations calculated using the associated Bio-Tek® KC4 microplate data analysis software. See Appendix I for detailed protocol.

DNA AP Site Assay

DNA abasic or apurinic/apyrimidinic lesions (DNA AP sites) are used as an indicator of genetic damage. The DNA AP site assay detects and quantifies a specific type of DNA lesion that can be generated by an alkylation or an oxidation event (Asaeda et al. 1998; Boturyn et al. 1999). DNA was isolated from frozen cryomilled tissue according to the manufacturer's instructions using the Dojindo Get *pure*DNA kit – Cell-Tissue (Cat# GK03-20; Dojindo Molecular Technologies, Inc., Rockville, MD) with one slight modification to address Maillard chemistry artifacts. The kit's lysis buffer was modified by the addition of approx. 5-10 mg of polyvinylpolypyrrolidone (PVPP, Sigma-Aldrich Corp. St. Louis MO). DNA was quantified with the Quant-iT™ High Sensitivity DNA kit (Cat# Q33120, Life Technologies) using a Qubit® fluorometer. DNA AP sites were quantified using the Dojindo DNA Damage Quantification Kit-AP Site (Cat# DK-02-12) and conducted according to the manufacturer's instructions, except for modifying the detection platform from the kit's colorimetric detection to chemiluminescence (Downs et al. 2006; Downs et al. 2011; May 2011).

Surveyor® Nuclease Mutation Detection Assay

During Task 1 of this project, the Surveyor® Mutation Detection assay for coral mitochondrial DNA (mtDNA) was adapted from the commercial human mutation detection diagnostic assay from Transgenomic®, Inc. (Omaha, NE). This development was undertaken as an alternative for the AFLP assay originally proposed, since our testing showed the AFLP assay was not suitable for these studies (see Appendix II for assay assessment report). We targeted mtDNA because of its importance in many biological functions, because mutations in this organelle's genome often have severe consequences, and for its propensity to have regions susceptible to mutation (e.g., D-loop). This assay recognizes mismatches in heteroduplexed DNA, which is made possible by the Surveyor® endonuclease. This nuclease cleaves both DNA strands on the 3' side of a mismatch, including base substitutions and insertion/deletions (up to 12 nucleotides). This provides the ability to survey large segments of DNA for numerous sequence variants. The method involves four major steps: 1) DNA isolation and quantification, 2) PCR amplification of targeted sequences, 3) heteroduplex formation, and 4) endonuclease digestion and agarose gel electrophoresis for visualization of DNA fragments (see Appendix III for detailed protocol).

DNA purification and quantification

Coral genomic DNA was isolated from approximately 60-80 mg frozen coral tissue homogenate using the Qiagen Genomic-tip 20/G kit (Life Technologies) according to the manufacturer's instructions. Genomic DNA was eluted in a total volume of 100 µL 10 mM Tris-1 mM EDTA (TE) buffer and 2 µL of the eluent was quantified with the Quant-iT™ High Sensitivity DNA kit using a Qubit® fluorometer. Samples (10 µL) were evaluated on a 0.5% agarose gel to verify integrity. Remaining DNA was aliquoted into sterile tubes (10 µL/tube) and stored at -20°C until use.

Amplification of coral mitochondrial DNA fragments

Eleven primer sets, which amplify overlapping 1.5-2.5 kb sections of the complete mitochondrial genomes of *Pocillopora damicornis* (GenBank accession number EF526302.1) or *Porites* sp. (GenBank accession numbers NC_015644.1 and DQ643837.1), were designed using coral mitochondrial genome sequences found in Genbank. Information on primer sets (A-K) for each species can be found in Tables 3 and 4. Oligonucleotides were tested to determine the optimal PCR conditions for each set.

Table 3. Oligonucleotide primers which amplify eleven overlapping regions of the *Pocillopora damicornis* mitochondrial genome.

Primer Name	Section	Sequence (5'-3')	Length (bp)	C+G	Tm	Ta	Product Size	Reference
Pdam mtDNA rnlF	A	GTT AGT ACA AAT AGT CCG TCG CC	23	47.8%	55 °C	60 °C	2.47Kb	this work
Pdam mtDNA nad5(5')R	A	CCA TTG CAT CCG GTA ACC AAG TAT G	25	48.0%	58 °C	60 °C		this work
Pdam mtDNA nad5(5')F	B	GGA GAT CCT CAT ATT CCT CGA TTT ATG	27	40.7%	57 °C	60 °C	1.96Kb	Flot and Tillier 2007, revised
Pdam mtDNA cobR	B	GTC CTC AAG GCA AAA CAT AGC CC	23	52.2%	57 °C	60 °C		this work
Pdam mtDNA cobF	C	CTT CAT GCT AAC GGT GCT TCT TTG	24	45.8%	56 °C	60 °C	2.28Kb	this work
Pdam mtDNA nad6R	C	CGC TGA AAC AAC CAT TAC TCC TGA GCC	27	51.9%	61°C	60 °C		this work
Pdam mtDNA nad2F	D	GCA GGA ATT CCT CCT TTT GCT GGC	24	54.2%	59 °C	60 °C	2.31Kb	this work
Pdam mtDNA orfR	D	CAC ACA TGA GCC ATC ATC CCT TC	23	52.2%	57 °C	60 °C		this work
Pdam mtDNA atp6F	E	CGC TAT TAG AGG TGG CAG TTG C	22	54.5%	57 °C	60 °C	2.19Kb	this work
Pdam mtDNA nad4R	E	GGA GCC TCA ACA TGT GCT TGT GGC	24	58.3%	61 °C	60 °C		this work
Pdam mtDNA nad4F	F	CAT CAT AGG GAC AGC GAG GGA GAG	24	58.3%	59 °C	60 °C	2.35Kb	this work
Pdam mtDNA cox3R	F	GAG AAG GCT CAA CCA AAT GAT A	22	40.9%	51 °C	60 °C		Flot and Tillier 2007
Pdam mtDNA rnsF	G	GTT AGT ACA AAT AGT CCG TCG CC	23	47.8%	55 °C	60 °C	1.48Kb	Flot and Tillier 2007
Pdam mtDNA cox2R	G	GCC CTA AAG AAG GCA CTG C	19	57.9%	53 °C	60 °C		this work
Pdam mtDNA cox2F	H	GAA GGG GAT ACG TTA GGG TTT G	22	50.0%	55 °C	60 °C	1.38Kb	this work
Pdam mtDNA nad5(3')R	H	CCG CAT GAA TAA GAG ACC CTG CAC TC	26	53.8%	61 °C	60 °C		this work
Pdam mtDNA nad3F	I	CCA GAT CGA GAA AAG GTT TCT GC	23	47.8%	55 °C	60 °C	2.14Kb	this work
Pdam mtDNA int11R	I	GAA CTG AAA GAC CCG CAT CTC CC	23	56.5%	59 °C	60 °C		this work
Pdam mtDNA atp8F	J	GCC ACA GTT AGA GGT AGG TAC	21	52.4%	54 °C	60 °C	2.38Kb	this work
Pdam mtDNA cox1R	J	CGT CTT GGA AAT CCT GCT AAA CC	23	47.8%	55 °C	60 °C		Flot and Tillier 2007, revised
Pdam mtDNA cox1F	K	CAA GCA CAC TCC GGA GGT TCT G	22	59.1%	59 °C	60 °C	2.51Kb	this work
Pdam mtDNA rnlR	K	CGC TAC ATT ATC ACA GTC AGT G	22	45.5%	53 °C	60 °C		this work

bp=base pair; C+G=percent cytosine and guanine bases in DNA primer sequences; Tm=melting temperature; Ta=annealing temperature

Table 4. Oligonucleotide primers which amplify eleven overlapping regions of the *Porites* sp. mitochondrial genome.

Primer Name	Section	Sequence (5'-3')	Length (bp)	C+G	Tm	Ta	Product Size	Reference
Porites mtDNA nad5(5')F	A	GGT GCT GGA ATT TTA ACT TCA AGC	24	41.7%	54°C	60°C	2.10 Kb	this work
Porites mtDNA cobR	A	GAT TCT CTT TGC GCA GTG GCA TAG G	25	52.0%	59°C	60°C		this work
Porites mtDNA nad1F	B	CGG TAT GAT CAG CTT ATG GCT C	22	50.0%	55°C	60°C	2.14 Kb	this work
Porites mtDNA nad2R	B	CAG ACC AGA TGA AAG TGC ACC	21	52.4%	54°C	60°C		this work
Porites mtDNA intF	C	GAT GGT GGA CAC GGA AAA GC	20	55.0%	54°C	60°C	2.08 Kb	this work
Porites mtDNA atp6R	C	CGA CAC CAT GAA GAT GAT CAT AG	23	43.5%	53°C	60°C		this work
Porites mtDNA nad6F	D	CTT GAG ATT TGG CAA CTC CTT GG	23	47.8%	55°C	60°C	2.28 Kb	this work
Porites mtDNA nad4R	D	CAA ACC CGT GCG CTA ACA TCA TG	23	52.2%	57°C	60°C		this work
Porites mtDNA nad4F	E	GCC TCC GAG TAT TTT GCT CCT C	22	54.5%	57°C	60°C	2.33 Kb	this work
Porites mtDNA cox3R	E	GGT CAA GCC ACA CCC AAT TCA AC	23	52.2%	57°C	60°C		this work
Porites mtDNA cox3F	F	GCG AAC TGT TTT ATG TCA TCC	21	42.9%	50°C	60°C	2.48 Kb	this work
Porites mtDNA nad5(3')R	F	GGA GCT TGT TCA AAA AGA GGA GAA G	25	44.0%	56°C	60°C		this work
Porites mtDNA nad3F	G	CTT TGG GTT TAC TCT ATG AGT G	22	40.9%	51°C	60°C	2.24 Kb	this work
Porites mtDNA cox1(5')R	G	CAT TGC ACC CAA AAT CGA GGA C	22	50.0%	55°C	60°C		this work
Porites mtDNA cox1(5')F	H	CTA CTA ACC ATA AAG ACA TTG GTA CG	26	38.5%	55°C	60°C	1.49 Kb	this work
Porites mtDNA coxintR	H	GAG CAC CCT TCT TCC CAC TAT GC	23	56.5%	59°C	60°C		this work
Porites mtDNA coxintF	I	CTA GGG TCA ATC AGT GGG AAA C	22	50.0%	55°C	60°C	2.13 Kb	this work
Porites mtDNA rnlR1	I	CTC GAC CTT CTC TTC ACC TAC	21	52.4%	54°C	60°C		this work
Porites mtDNA trnMF	J	GTA GAG AAG ACG AAT GGT GAG TC	23	47.8%	55°C	60°C	2.19 Kb	this work
Porites mtDNA rnlR2	J	GAA ACC AAG CTG TGT TAC CAC GC	23	52.2%	57°C	60°C		this work
Porites mtDNA rnlF	K	GCT TGG TAG TAG AAC AGA CTG	21	47.6%	52°C	60°C	2.16 Kb	this work
Porites mtDNA nad5(5')R	K	CCA ACT GTG CAG ACT TTC CAA CC	23	52.2%	57°C	60°C		this work

Once primer sets were optimized and products verified, they were used to amplify samples of munitions-treated coral. Replicate coral fragments from four treatments (carrier control, lowest dose, mid-range dose, and highest dose) were amplified with each primer set. One carrier control replicate was amplified in duplicate to provide the additional product required for the assay uncut control, and a negative control was included for a total of ten reaction tubes per primer set. The PCR mixture used to assay munitions-treated coral in this project included: 6-8 ng template DNA, 1 µL 10 mM dNTPs, 20 pmol each primer, 0.25 µL ExTaq™ polymerase (TaKaRa), 5 µL 10 X ExTaq™ buffer and sterile water to 50 µL. Cycling parameters were: 94°C for 5 min; 35 cycles of: 94°C for 30 s, 60°C for 30 s, 72°C for 3 min; and a final extension at 72°C for 10 min. Products (5 µL) were evaluated on a 1.2% agarose gel to ensure that no secondary amplification products were present. Following amplification, products were quantified using the Qubit® fluorometer as described above. Products were stored at -20°C until use, but for no longer than 3 days. Surveyor® Plasmid Control C and Plasmid Control G PCR products were prepared as directed in the Suveyor® Nuclease Mutation Assay kit protocol. Amplification products were quantified as described above and stored at -20°C until use.

Heteroduplex Formation

Coral mitochondrial DNA PCR products from a munitions dosing experiment were placed in 0.2 mL thin-walled tubes. Briefly, one carrier control replicate DNA sample (150 ng) was mixed with an equal amount of treated coral sample to be assayed, in a final volume of 12 µL. In addition to the heteroduplex mixes, two carrier control tubes (300 ng each) were included for uncut and digested homoduplex controls. Also, one tube containing 300 ng of Plasmid Control C PCR product (homoduplex) and a second tube containing 150 ng of Plasmid Control C and 150 ng Plasmid Control G PCR products (heteroduplex) were prepared for nuclease assay validation. Sample tubes were placed in a thermocycler and DNA heteroduplexes were formed using the cycling parameters described in the kit protocol. Heteroduplex PCR products were immediately treated with Surveyor® Nuclease to determine the number of base pair mismatches (mutations) per sample.

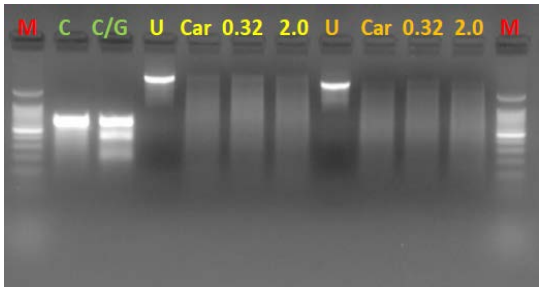


Figure 12. Results from initial testing of two RDX-treated *Pocillopora damicornis* mtDNA sections using manufacturer's recommended Surveyor® Nuclease Assay conditions. Coral DNA was completely digested. Lanes: M=100 bp DNA ladder, C= Plasmid Control, C/G=Plasmid with single base pair mismatch, U=Uncut coral mtDNA section, Car=coral control homoduplex, 0.32=0.32 µg/mL RDX heteroduplex, 2.0=2.0 µg/mL RDX heteroduplex. Yellow=mtDNA Section J and Orange=mtDNA Section B.

Surveyor® Nuclease Assay

Initial testing of coral mtDNA heteroduplexes indicated that the manufacturer's suggested assay conditions for the nuclease treatment completely digested the PCR products (Fig. 12). Variable time and nuclease concentration tests were performed to optimize the experimental parameters for the coral mtDNA sections (Fig. 13). The experimentally determined optimal conditions (20 min incubation at 42°C, 0.5 µL nuclease) were used for all munitions-treated coral samples. Plasmid control reactions were executed with the kit suggested protocol (60 min incubation at 42°C, 1.0 µL nuclease). The complete reaction volume for the assay also included 1.0 µL Enhancer S (kit provided) for all samples. Following incubation, 1.5 µL Stop Solution (kit provided) was added to each tube and samples were mixed by gently pipetting up and down. A 3.5 µL aliquot of 6 X tracking dye was mixed into each tube and the samples were loaded onto a 2.0 % TAE-agarose gel containing 0.25 µg/mL ethidium bromide. Samples were electrophoresed for 2 h at 50 volts prior to imaging.

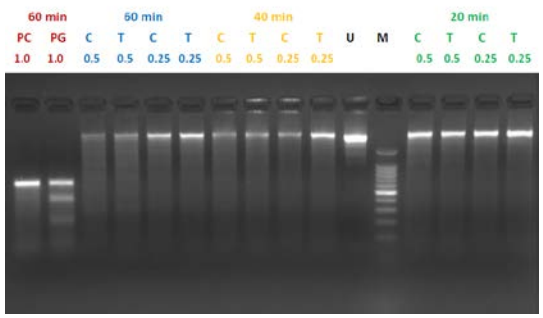


Figure 13. Results of nuclease concentration and incubation time test experiment using 0.25-1.0 µL enzyme and 20-60 min incubations on coral mtDNA amplification products. Coral samples digested with 0.5 µL nuclease for 20 min at 42°C gave optimal results. Lanes: PC=plasmid control, PG=plasmid mutation control, C=homoduplex control samples, T=heteroduplex control + treated samples, U=uncut coral control and M=100 bp DNA markers. Bottom numbers indicate the µL of Surveyor® Nuclease added to each reaction mixture. Incubation times (42°C) are indicated at the top of the gel.

Physio-scoring Coral Fragments

Corals were visually scored daily for signs of adverse health effects using a set of characters associated with health condition. The scoring criteria were refined to distinguish gradations of change in tissue integrity, color and polyp behavior. For tissue, ≥ 75% tissue loss was scored as 1, 50% tissue loss as 2, 25% tissue loss as 3, tissue thinning scored as 4 and healthy intact tissue as 5. Color was scored as bleached or no color but tissue present as 1, paling as 3 and healthy, normal color as 5. Polyp behavior was scored with the following criteria, full retraction=1; partial retraction=3 and full extension=5 (Fig. 14).

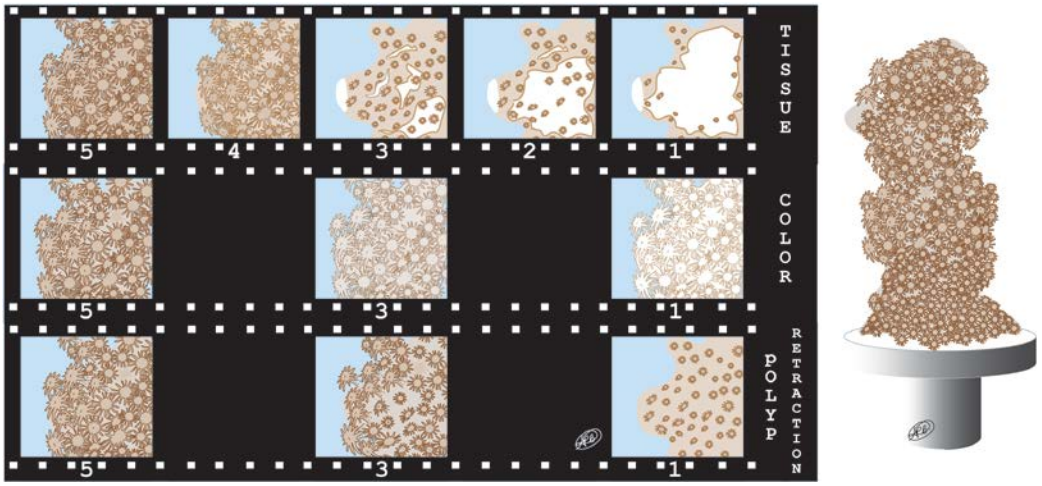


Figure 14. Physio-scoring analysis. Criteria used for *Pocillopora damicornis* evaluation was based on three morphological features that can change with varying health conditions in coral; these are tissue behavior, color and polyp behavior. These were visually scored using a 1-5 scale, with 5 as the optimal condition. *Artwork by Athena R. Burnett.*

Histology

Coral fragments were taken from munitions dosing experiments at designated time points and placed into 5-10 mL of zinc-formalin fixative (Z-fix) in SSS-ASW. The Z-fix solution consists of the following: 20% (v/v) of concentrated Zinc-formalin (Cat# 171, Anatech, LTD, Battle Creek, MI) 20% (v/v) of 2x SSS-ASW at 70 ppt, and 60% (v/v) of 1x SSS-ASW at 35 ppt for at least 72 h. Fixed samples were placed into labeled tissue cassettes, rinsed three times with SSS-ASW (35 ppt), and then into decalcification solution (0.3M EDTA pH 7.5-8.0). Samples were gently mixed by swirling the container intermittently throughout the workday. The decalcification solution was replenished every 2-3 days. Depending on the size and density of the coral fragment, decalcification can take a few days to 2 weeks; however, the decalcification process should be monitored and samples removed from the decalcification solution and processed as soon as skeleton dissolution is complete. The sample decalcification is complete when the tissue is soft when gently touched with a probe. After decalcification the samples were rinsed (3X) in ASW (35 ppt) and dehydrated (1-24 h each step) through a graded series of

ethanol (50-100%). Samples were stored in 70% histological grade ethanol until processed (3.5 h processing program on automatic tissue processor) into a paraffin block. Paraffin blocked samples were serially sectioned at 5 microns using a rotary microtome (Leica RM225), placing 3 to 10 sections per slide. Slides were then stained with hematoxylin-eosin for light microscopic examination.

Transmission Electron Microscopy

To examine the ultrastructure of coral tissue, an approximate 1 cm subsample was removed from the experimentally dosed coral fragment. For primary fixation, the sample was submerged in modified Karnovsky's fixative (2.5% gluteraldehyde, 2% paraformaldehyde in 0.1M cacodylate buffer (pH 7.2) for 30 min and then transferred to a solution of 2.5% gluteraldehyde in filtered seawater. Samples were fixed overnight in this secondary fixative. For transportation, gluteraldehyde-fixed samples were placed into 1% gluteraldehyde in cacodylate buffer at 4°C until final processing and electron microscopy. To complete the processing, the samples were washed three times for 15 min each in 0.1 M cacodylate buffer (pH 7.2) that was adjusted with NaCl to seawater molarity and pH. The samples were then decalcified with 10% Na_2EDTA, pH 7.5-8.0 by first replacing the cacodylate wash buffer with the 10% EDTA solution and rinsing for 15 min. This was replaced with a fresh EDTA solution every 12–24 hours until the tissue was decalcified. The samples were then post-fixed in 1% osmium tetroxide at room temperature for 1 h, to enhance membrane preservation, and rinsed in buffer. Samples were dehydrated in a graded ethanol series, then in propylene oxide followed by gradual embedding in Embed 812 (Electron Microscopy Sciences, Fort Washington, PA). Samples in the final, full-strength Embed 812 were subjected to a mild vacuum (400 mbar) for 1 h at 25°C followed by 48 h of polymerization at 60°C. The resulting block was trimmed and 1 micron sections were cut and stained with toluidine blue (Carson 1997). The block was then sectioned (60–90 nm) using an ultra-microtome and sections mounted onto 300 mesh copper grids. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections through approximately the same mid-polyp body area were examined using a JOEL JEM-1010 at 80 kV transmission electron microscope and images photodocumented with a Hamamatsu camera (Hamamatsu Photonics, Japan).

Method Brief – Metrology of Munitions Compounds

A LC-MS method was developed for the detection of munitions compounds in treatment waters and to determine the behavior of these chemicals (i.e., breakdown) in the experimental systems. A detection method was optimized for five munitions compounds and eleven degradation or metabolic by-products in the low nanogram range (Table 5). Standards of native compounds in 50:50 methanol (MeOH):acetonitrile (AcCN) were purchased from AccuStandard (New Haven, CT); the EPA 8330-R mixture was the primary component, to which was added the five munitions compounds. Isotopically labeled standard solutions ($^{13}C_3$-RDX in AcCN, $^{13}C_7$, $^{15}N_3$-

TNT in benzene, and D_3-2,6-DNT in AcCN for internal standards and $^{13}C_3$-HMX in AcCN for recovery standard) were purchased from Cambridge Isotope Laboratories (Andover, MA). An intermediate stock solution of target compounds at 1000 ng/mL was used for method optimization. Two calibration curves (0.5-1000 ng/mL [9 points modified geometric]), spiking stock (100 ng/mL), internal standard (5000 ng/mL), and recovery standard (20000 ng/mL) solutions were prepared volumetrically at room temperature in 50:50 MeOH:reagent grade water (H_2O) to best match the mobile phase.

Table 5. Compound, detection, and calibration parameters for LC-MS of munitions compounds.

Analyte	RT (min)	Quantification[1]		Range (ng[2])	Pearson r^2	DP, EP, CE, CXP[3]
4-Hydroxylamino-2,6-dinitrotoluene	4.5	UV	254 nm	5 - 1000	1.000	N/A
2,6-Diamino-4-nitrotoluene	6.4	UV	254 nm	25 - 1000	0.999	N/A
2,4-Diamino-6-nitrotoluene	7.4	UV	254 nm	25 - 1000	0.999	N/A
HMX	8.6	MRM	371.1-146.1	0.5 - 1000	0.999	-25, -5, -10, -10
RDX	12.8	MRM	267.1- 44.9	0.5 - 1000	0.999	-25, -5, -30, -15
Tetryl	17.0	MRM	318.1-120.1	1.0 - 500	0.995	-25, -5, -40, -20
TNT	18.2	MRM	226.1- 63.1	0.5 - 1000	0.992	-50, -5, -40, -10
4-Amino-2,6-dinitrotoluene	18.9	MRM	196.1-119.1	0.5 - 1000	0.997	-50, -15, -20, -10
2-Amino-4,6-dinitrotoluene	19.4	MRM	196.1-135.9	0.5 - 1000	0.997	-50, -10, -20, -10
2,6-Dinitrotoluene	19.7	UV	254 nm	10 - 1000	1.000	N/A
2,4-Dinitrotoluene	20.0	UV	270 nm	5 - 1000	1.000	N/A
PETN	20.8	MRM	391.1- 61.1	0.5 - 1000	0.999	-50, -10, -20, -10
2-Nitrotoluene	21.1	UV	254 nm	10 - 1000	1.000	N/A
4-Nitrotoluene	21.5	UV	270 nm	5 - 1000	1.000	N/A
3-Nitrotoluene	22.0	UV	270 nm	5 - 1000	1.000	N/A
2,2',6,6'-Tetranitro-4,4'-azoxytoluene	28.4	MRM	405.3-196.3	0.5 - 1000	0.998	-50, -5, -30, -10

1 UV: absorbance at the listed wavelength; MRM: *m/z* transition

2 Final units depend on amount of material extracted/concentrated

3 meaningful only for MRM

N/A – Not Applicable; DP – Declustering Potential; EP – Exit Potential; CE – Collision Energy; CXP – Cell Exit Potential

An Agilent 1100 Series HPLC with binary pump, thermostatted autosampler, heated column compartment (at 25°C), and photodiode array detector (off for this method) was used to introduce a 20 µL injection to an AB Sciex (Framingham, MA) API4000 tandem mass spectrometer (MS/MS) with an electro-spray ionization source operating in negative polarity (ESI-) with scheduled multiple reaction monitoring (MRM) program; detector parameters for each transition were optimized using automated flow injection analysis. Gas and scan parameters were the same for all compounds (CAD=10, CUR=10, GS1=40, GS2=50, IS=-4500, TEM=300,

ihe=Off, Scan Time=2s, MRM Window=360s). Separation was achieved in 30 min (Fig. 15) using a Phenomenex (Torrence, CA) Synergi 4µ Hydro-RP 80A analytical column (fitted with matching guard column) with a methanol-water gradient at 1 mL/min with a maximum back pressure of 354 bar (Table 6). Internal standards (100 ng) and recovery standard (100 ng) were added to the calibration curve for MS/MS analysis.

Table 6. Mobile phase gradient.

Time (min)	Water (%)
0	60
2	60
30	0
32	60
40	60

Several analytes did not ionize well in either ESI or atmospheric pressure chemical ionization sources, likely due to nitro-group shielding. The same HPLC program and column (with both visible and UV lamps on) was used for quantification of the remaining target analytes by absorbance at 254 or 270 nm using a 100 µL injection (Fig. 15). A spectral absorbance library was built to aid in identification and purity analyses. The second calibration curve, lacking internal and recovery standards, was used for this analysis; D_3-2,6-DNT was used

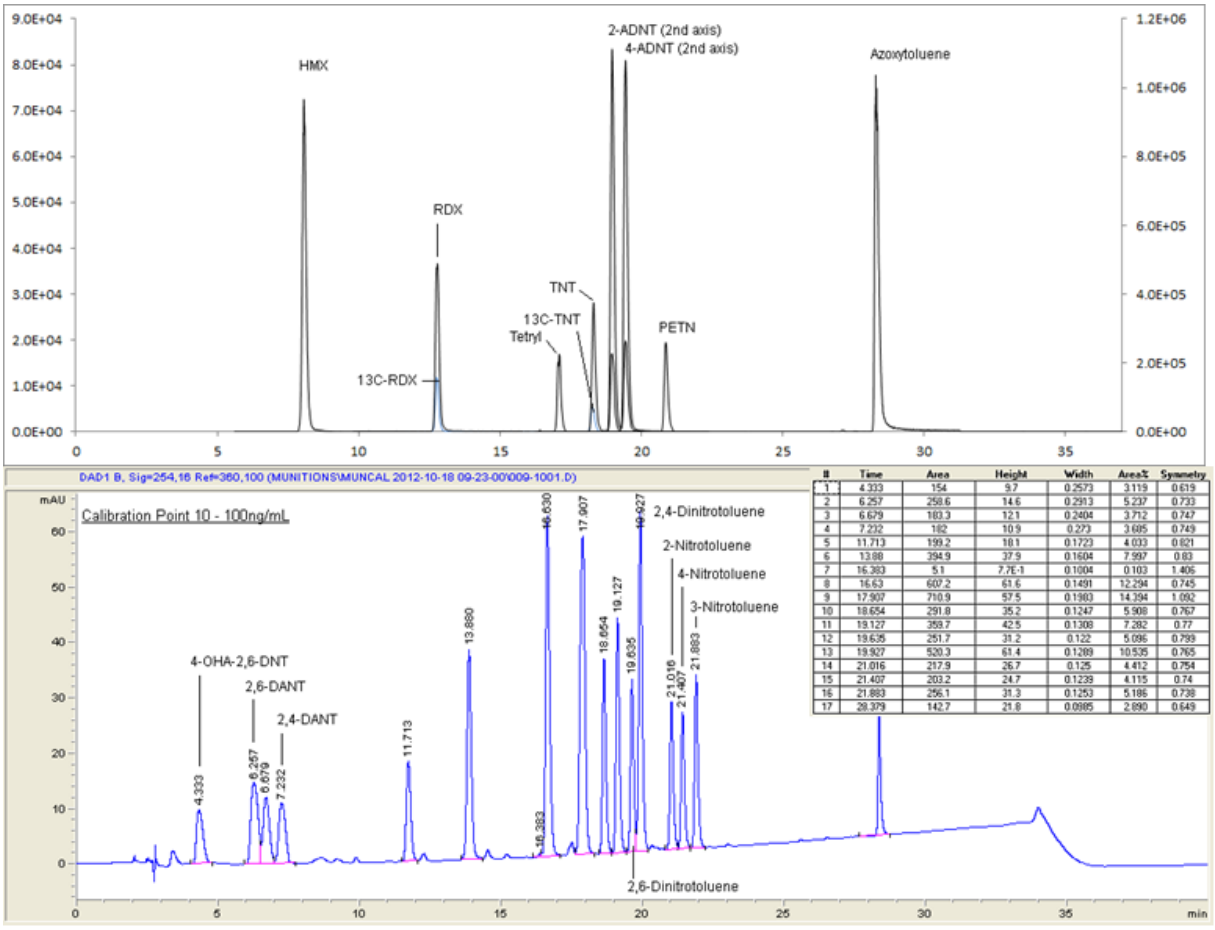


Figure 15: Example chromatogram of 19 munitions compounds and degradation/metabolic byproducts for (a) MRM and (b) UV.

instead as an external standard addition for samples. For toxicity screens well above the detection range of the method, samples were vortexed ≥15s and an aliquot diluted to within the target range with 50:50 MeOH:H_2O. To each aliquot was added 20 µL of internal standard solution (for 10 ng) and the resulting solution vortexed ≥5s; aliquots were standardized to 1 mL final volume. Samples were then passed through a nylon 0.45 µm syringe filter and 5 µL of recovery standard solution (for 10 ng) was added with a final vortex ≥5s. Processed samples were stored in the dark at 4°C until analysis. All vials were vortexed ≥5s prior to injection.

Statistical Methods

Mortality tests

Median lethal concentration (LC_{50}) values and confidence intervals were calculated by the PROBIT procedure (PROC Probit - SAS Version 9.4, SAS Institute, Cary, NC, USA) following the recommendations by Newman (1995) using either a normal, logistic, or a Gompertz distribution. Nominal concentrations were used in the analyses. If the data did not fit the PROBIT model (as determined by a Chi-Square Goodness of Fit test, $p<0.05$), then the Trimmed Spearman-Karber approach was used (Hamilton et al. 1977 and Newman 1995) in addition to the PROBIT as a confirmatory analysis.

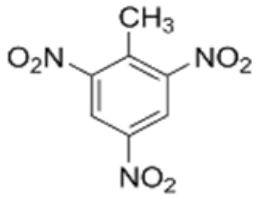
The No-Observed Effects Concentration (NOEC) and Lowest-Observed Effects Concentration (LOEC) values were determined by comparing mortality in the treatments to mortality in the controls. The NOEC is defined as the highest concentration that was not significantly different than the control group. The LOEC is defined as the lowest concentration that was significantly different than the control group. For this, a one-way Analysis of Variance (ANOVA) with Dunnett's test for multiple comparisons versus control was used (Zar 1999, PROC GLM, SAS Version 9.4, SAS Institute, Cary, NC, USA). When all-pairwise comparisons were of interest, the ANOVA was followed by the post-hoc Tukey HSD multi-comparison test (Zar 1999). The assumptions of ANOVA, normality and homogeneity of variances, were checked by looking at the Shapiro-Wilk test and the Levene's test respectively in addition to graphical examination of residual plots. While mild to moderate departures from these assumptions ($0.05 > p > 0.001$) were found to be acceptable in many cases (Zar 1999), a nonparametric ANOVA (Kruskal-Wallace test) with a post-hoc nonparametric multiple comparison was used (Zar 1999) when departures were moderate to severe ($p<0.001$).

Sub-lethal tests

Median effect concentration (EC_{50} / IC_{50}) values were determined by fitting a 3-parameter logistic equation to the data as described by Martikainen and Krogh (1999). Parameter estimations and their corresponding confidence intervals were calculated using PROC NLIN (SAS Version 9.4, SAS Institute, Cary, NC, USA). Significant differences between treatment groups and controls were performed using the same statistical procedures (ANOVA with Dunnetts and/or Tukey) as described previously. Alpha (α) for all statistical tests was set to 0.05 *a priori*.

RESULTS AND DISCUSSION

2,4,6-Trinitrotoluene



2,4,6-TNT

Background

TNT (CAS# 118-96-7; IUPAC, 2-methyl-1,3,5-trinitrobenzene, also called 2,4,6-trinitrotoluene; MW: 227.13 g/mol; color/form: pale yellow, loose needles, flakes, prills) is a nitrotoluene and among other nitroaromatic compounds, primarily used in the explosives industry. The solubility in seawater was determined by Prak and O'Sullivan (2006) to be 86 mg/L at 20°C and 121 mg/L at 30°C. Use, storage, improper disposal and incineration can all introduce the explosive into the water column (Nipper et al. 2001). An extensive scientific literature documents its toxicity in humans and other mammals, affecting the hematopoietic and hepatic systems predominantly, and in avian species the effects predominate in the central nervous system. TNT also shows extensive toxicity in aquatic organisms including vertebrates, invertebrates, and plants, which affects growth, development and reproduction. TNT exhibits genotoxicity across all species that have been tested. Nipper et al. (2001; 2009) reported the toxicity values for multiple marine organisms including the marine alga (*Ulva fasciata*) zoospore germination, polychaete (*Dinophilus gyrociliatus*) survival, sea urchin (*Arbacia punctulata*) embryo development, and redfish (*Sciaenops ocellatus*) embryo-larval survival. The EC_{50} values were determined as 2.5 mg/L for *Ulva*, 7.7 mg/L for the polychaete, 12 mg/L for the sea urchin and 8.2 mg/L for the redfish. Nipper and O'Sullivan (2001) also determined the NOEC/LOEC values for these marine organisms: *Ulva* 1.4/2.9 mg/L, polychaete 6.1/11.6 mg/L, sea urchin 9.1/19 mg/L, and redfish 5.4/10.3 mg/L. One additional feature of nitroaromatic compounds, including TNT, is a reaction process called photoactivation, also called photo-enhanced or photo-induced toxicity. This results in enhancement of toxic effects when the susceptible compounds are exposed to UV light. Photoactivation, however, does not appear to affect all organisms. TNT is also subject to another type of reaction, photo-transformation. This occurs in the presence of near-UV (336-400 nm) light and involves the breakdown of the parent compound into other toxic compounds (e.g., TNT>2,4DNT, 2,6DNT).

Because of the extensive use of TNT in munitions and its known toxicity in a wide range of organisms, tests were conducted with coral cells, isolated coral symbionts and coral fragments. Coral cells were from two coral species (*Pocillopora damicornis* and *Porites divaricata*) and included two cell types from each species (calicoblasts and algal-symbiont-containing gastrodermal cells). Because of TNT's photoreactive characteristics, coral cell toxicity assays included treatments in both light and dark conditions under varying concentrations of TNT. In addition and as supportive evidence for the coral cell toxicity assay, *Symbiodinium* sp. Clade B cell cultures established from *Pocillopora damicornis* were also tested as a representative of the effects on the coral symbiont alone without interactions from its cnidarian host. Intact coral fragments from *Porites divaricata* were also subjected to a 96 h exposure with varying

concentrations of TNT and examined for sublethal indicators of toxicity (histology, DNA AP sites, and porphyrin).

Coral Cell Toxicity Assays

Cell Response

Primary cells were isolated and cultured as described in the methods and plated into 24-well Teflon® plates at approximately $6.5x10^5$ to $1.0x10^6$ cells per well. Cells were exposed for 4 h in the light (295 µmol $m^{-2}s^{-1}$) or darkness at 25°C in media containing concentrations of TNT ranging from 0.5 µg/L to 50,000 µg/L. Cell counts were performed on each well at the initiation of the exposure and at the end of 4 h using an exclusion dye viability assay.

During the course of these experiments, several unexpected effects were observed. The first occurred during the cell viability assays. It was observed that with increasing munitions exposure concentrations, there appeared to be a loss in the number of total cells compared to numbers at the initiation of the experiment (4 h earlier) that could not be accounted for based on technical error or imprecision. It was determined that none of the five exclusion dyes (trypan blue, Evans blue, neutral red, Naphthol blue-black, amido black) stained 100%. This means that in a population of dying cells, this assay, which is based on disruption of cell membranes to allow dye into cells, has limitations based on the extent of cellular disruption. This conclusion was based on the ability to detect autofluorescence remaining in coral cells severely disrupted, while not staining well with any of the viability exclusion dyes normally used to assess cell death. The second finding was that the concentration of an exposure compound, exposure time and the severity of cell death affect the performance of colorimetric vital stains (Fig. 16).

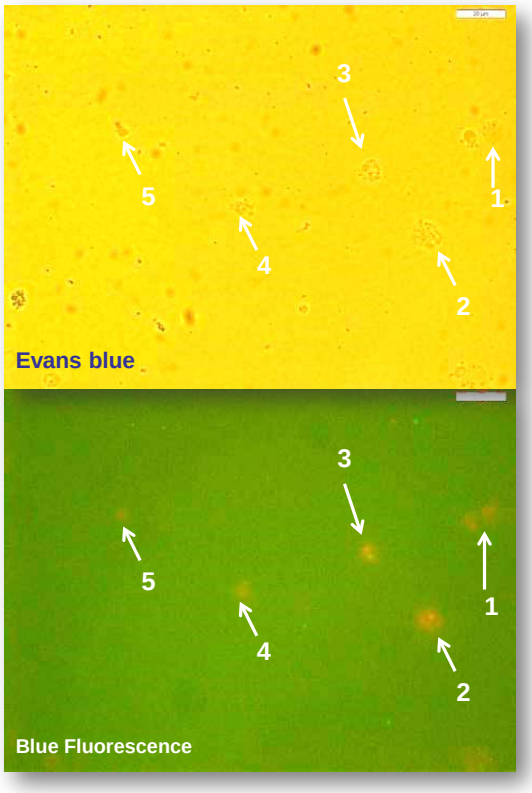


Figure 16. *Pocillopora damicornis* calicoblast cells showing reduced vital dye staining. Top panel is an example of reduced staining of cells with Evans blue vitality stain (arrows). Bottom panel shows autofluorescence of the same coral cells (arrows) from the top panel. *Photos by Craig Downs*.

In addition to the noticeable reduction in the uptake of vitality stains with higher TNT treatment levels, a second unexpected observation occurred with the gastrodermal cells, which host the algal symbiont, *Symbiodinium* sp. After a 4 h exposure in the dark, the gastrodermal cells and their dinoflagellate symbionts were observed rupturing and extruding their cellular contents within 1 min of viewing under bright-field microscope illumination. The rupturing of cells and chloroplasts within the gastrodermal cells was evidenced dramatically under conditions that excites chlorophyll fluorescence. This could be

viewed in real time occurring (Fig. 17) as a light-induced photo-associated toxicity. Cells began to rapidly rupture when exposed to TNT concentrations >100 µg/L in the dark and then illuminated under the white microscope lighting. It is not clear if this observation was a result of photo-enhancement or photo-induced toxicity once the pre-exposed cells were introduced into the light. To determine the mechanisms underlying these observations will require further study. It will also be important to determine if the effects observed in primary cells also occur *in vivo* in the intact coral tissue. The rupturing of the gastrodermal cells and their zooxanthellae is consistent with anecdotal information from the field of coral bleaching in areas with leaking munitions from ordnance that had breached.

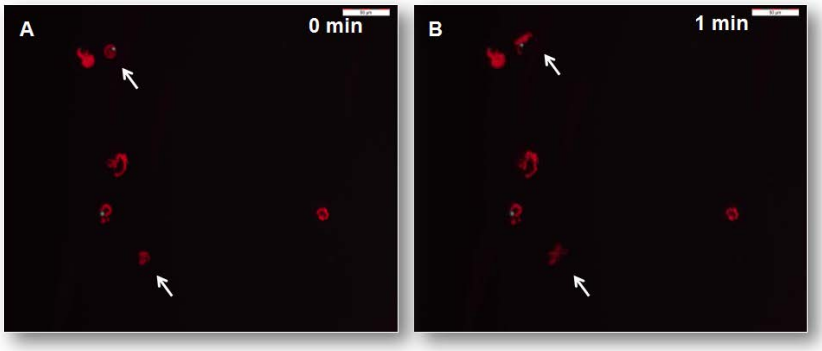


Figure 17. *Pocillopora damicornis* gastrodermal cells shown rupturing under fluorescence excitation after TNT exposure. A) Chlorophyll fluorescence of the algal symbiont (red fluorescing cells) within the gastrodermal cells at initial viewing under fluorescent microscope. B) Chlorophyll fluorescence of the algal symbiont within the gastrodermal cell after 1 min exposed to the blue excitation light of the microscope. White arrows mark the same cells at time 0 and 1 min later. *Photos by Craig Downs*.

Observed-effect concentrations (NOEC and LOEC)

An eight-point concentration gradient from 0.5 to 50,000 µg/L of TNT was used for the exposures of calicoblast and gastrodermal cells from *Pocillopora damicornis* (Indo-Pacific branching species). A nine-point concentration gradient from 0.1 µg/L to 50,000 µg/L was used for exposures of calicoblast and gastrodermal cells from *Porites divaricata* (Caribbean branching species) exposed in the light and a ten-point concentration gradient from 0.1 to 75,000 µg/L was used for exposure of calicoblast and gastrodermal cells from *P. divaricata* in dark conditions. Primary cultures of calicoblasts and gastrodermal cells were isolated from each species and cultured, then re-purified and aliquoted into 24-well Teflon® plates at approximately $6.5x10^5$ to $1.0x10^6$ cells per well. Cells were exposed for 4 h in light or dark conditions at 25°C. These conditions were used to reduce possible confounding effects due to photo-degradation or photo-activation that can occur with these compounds. Methanol was used as the carrier solvent for this compound. Cell viability counts were performed on each well using the trypan blue exclusion dye viability assay as the endpoint for effects characterization.

Pocillopora damicornis calicoblast and gastrodermal cells were exposed to various concentrations of TNT for 4 h in light or dark conditions (Fig. 18 and Table 7). A significant effect on percent mortality ($p<0.0001$) was found for the **calicoblast cells exposed in the light** as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison found a

significant ($p<0.05$) difference between control versus treatments. The NOEC was <0.5 µg/L (<500 pptrillion) and the LOEC was 0.5 µg/L (500 pptrillion) (Fig. 18A).

Pocillopora damicornis gastrodermal cells exposed to TNT in the light. A significant effect on percent mortality ($p<0.0001$) was found for the **gastrodermal cells exposed in the light** as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison found a significant ($p<0.05$) difference between control versus treatments. The NOEC was 0.5 µg/L (500 pptrillion) and the LOEC was 5 µg/L (5 ppb) (Fig. 18C).

Pocillopora damicornis **gastrodermal cells exposed to TNT in the dark.** Viability counts at concentrations above 100 µg/L were not reliable due to cells rupturing when exposed to the white light of the bright-field microscope during counting; these concentrations were therefore excluded from analysis (see Fig. 18D red stars). However, a significant effect on percent mortality ($p<0.0001$) was found for *P. damicornis* gastrodermal cells from the remaining treatment concentrations, as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison test for the remaining treatments versus a control were found to be significant ($p<0.05$) for mortality. The NOEC was 0.5 µg/L (500 pptrillion) and the LOEC was 5 µg/L (5 ppb) (Fig. 18D; Table 7).

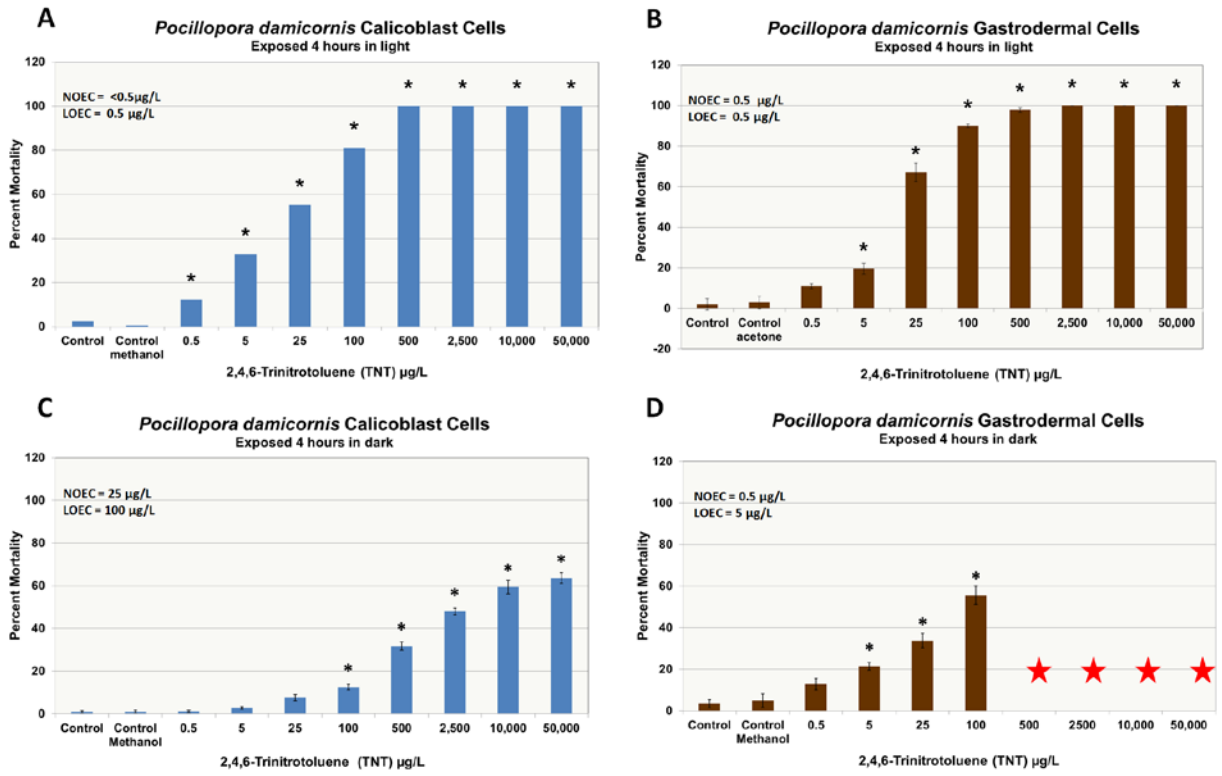


Figure 18. Percent mortality of *Pocillopora damicornis* cells exposed to various concentrations of TNT in light and dark conditions. n= 4 replicates per treatment. Bars show treatment means with ±1 SEM. Treatment means with * differed significantly at $\alpha = 0.05$, one-way ANOVA followed by Dunnett's test for multiple comparisons versus the carrier control. A) Calicoblast cells exposed in light. B) Gastrodermal cells exposed in light. C) Calicoblast cells exposed in darkness. D) Gastrodermal cells exposed in darkness. NOEC = no-observed-effect concentration. LOEC = lowest-observed-effect concentration. Red stars indicate samples with cells that ruptured when exposed to the white light of the microscope while counting.

Porites divaricata calicoblast and gastrodermal cells were exposed to various concentrations of TNT for 4 h in light and dark conditions (Fig. 19 and Table 7). A significant effect on percent mortality ($p<0.0001$) was found for the **calicoblast cells in light** as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison test for treatments versus a control found all concentrations at 500 µg/L and greater were significantly ($p<0.05$) higher for mortality than the control group. The NOEC was 100 µg/L (100 ppb) and the LOEC was 500 µg/L (500 ppb) (Fig. 19A). A significant effect on percent mortality ($p<0.0001$) was found for the **calicoblast cells in darkness** as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison test for treatments versus a control also found that all concentrations at 100 µg/L and greater were significantly ($p<0.05$) higher for mortality than the control group. The NOEC was 100 µg/L (100 ppb) and the LOEC was 500 µg/L (500 ppb) (Fig. 19C).

Porites divaricata gastrodermal cells responded in a dose-dependent manner throughout all concentrations. A significant effect on percent mortality ($p<0.0001$) was found for *Porites divaricata* **gastrodermal cells exposed in the light** to TNT as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison for treatments versus a control found all concentrations at 100 µg/L and greater were significantly ($p<0.05$) higher for mortality than the control group. The NOEC was 25 µg/L (25 ppb) and the LOEC was 100 µg/L (100 ppb) (Fig. 19B). A significant effect on percent mortality ($p<0.0001$) was found for *P. divaricata*

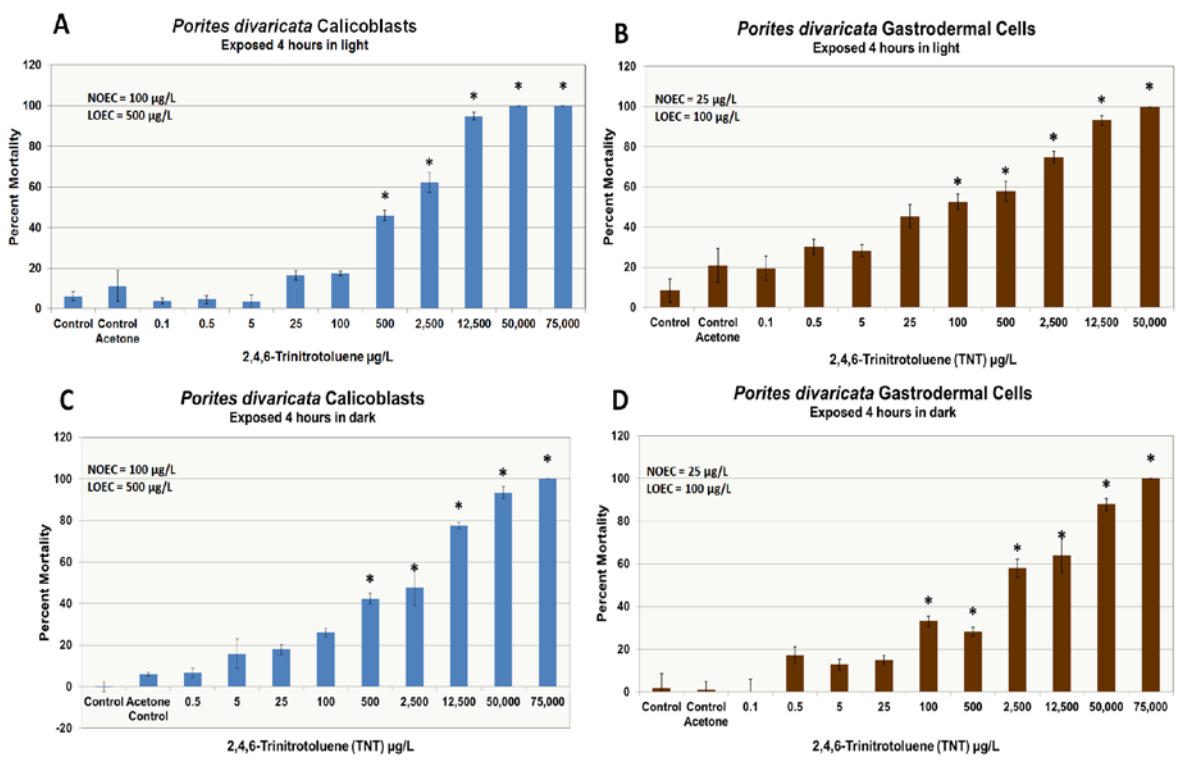


Figure 19. Percent mortality of *Porites divaricata* cells exposed to various concentrations TNT in light and dark conditions. n=4 replicates per treatment. Bars show treatment means with ±1 SEM. Treatment means with * differed significantly at $\alpha = 0.05$, one-way ANOVA followed by Dunnett's test for multiple comparisons versus the carrier control. A) Calicoblast cells exposed in the light. B) Gastrodermal cells exposed in the light. C) Calicoblast cells exposed in darkness. D) Gastrodermal cells exposed in darkness. NOEC = no-observed-effect concentration. LOEC = lowest-observed effect concentration.

gastrodermal cells exposed in darkness to TNT as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison test for treatments versus control found all concentrations at 100 µg/L and greater were significantly ($p<0.05$) higher for mortality than the control group. The NOEC was 25 µg/L (25 ppb) and the LOEC was 100 µg/L (100 ppb) (Fig. 19D).

These results demonstrate differences in cellular responses to TNT exposure, depending on species, cell type and lighting. *Pocillopora damicornis* in general displayed more sensitivity to TNT exposure than *P. divaricata*, with *P. damicornis* gastrodermal cells being the most sensitive. For *P. divaricata*, gastrodermal cells appeared more sensitive than calicoblast cells (NOEC 25 µg/L vs 100 µg/L, respectively).

LC_{50} and LC_{20}

Pocillopora damicornis and *Porities divaricata* calicoblast and gastrodermal cell mortality data for TNT were subjected to PROBIT analysis to determine the median lethal concentration (LC_{50}) and 20% lethal concentration (LC_{20}) values and confidence intervals.

The LC_{50} for ***Pocillopora damicornis*** **calicoblast cells exposed in light** was 16 µg/L (16 ppb) and the LC_{20} was 1.85 µg/L (1.85 ppb). The LC_{50} for ***Pocillopora damicornis*** **calicoblast cells exposed in darkness** was 1582 µg/L (1582 ppb) and the LC_{20} was 200 µg/L (200 ppb) (Fig. 20A&B; Table 8).

The LC_{50} for ***Pocillopora damicornis*** **gastrodermal cells exposed in light** was 15.3 µg/L (15.3 ppb) and the LC_{20} was 2.15 µg/L (2.15 ppb). The LC_{50} for ***Pocillopora damicornis*** **gastrodermal cells exposed in darkness** was 140 µg/L (140 ppb) and the LC_{20} was 12.7 µg/L (12.7 ppb) (Fig. 20 C&D; Table 8).

The LC_{50} for ***Porites divaricata*** **calicoblast cells exposed in light** was 716 µg/L (716 ppb) and the LC_{20} was 60.5 µg/L (60.5 ppb). The LC_{50} for ***Porites divaricata*** **calicoblast cells exposed in darkness** was 968 µg/L (968 ppb) and the LC_{20} was 32.6 µg/L (32.6 ppb) (Fig. 21A&B; Table 8).

The LC_{50} for ***Porites divaricata*** **gastrodermal cells exposed in light** was 54 µg/L (54 ppb) and the LC_{20} was 0.36 µg/L (360 pptrillion). The LC_{50} for ***Porites divaricata*** **gastrodermal** cells **exposed in darkness** was 1,196 µg/L (1,196 ppb) and the LC_{20} was 21 µg/L (21 ppb) (Fig. 21C&D; Table 8).

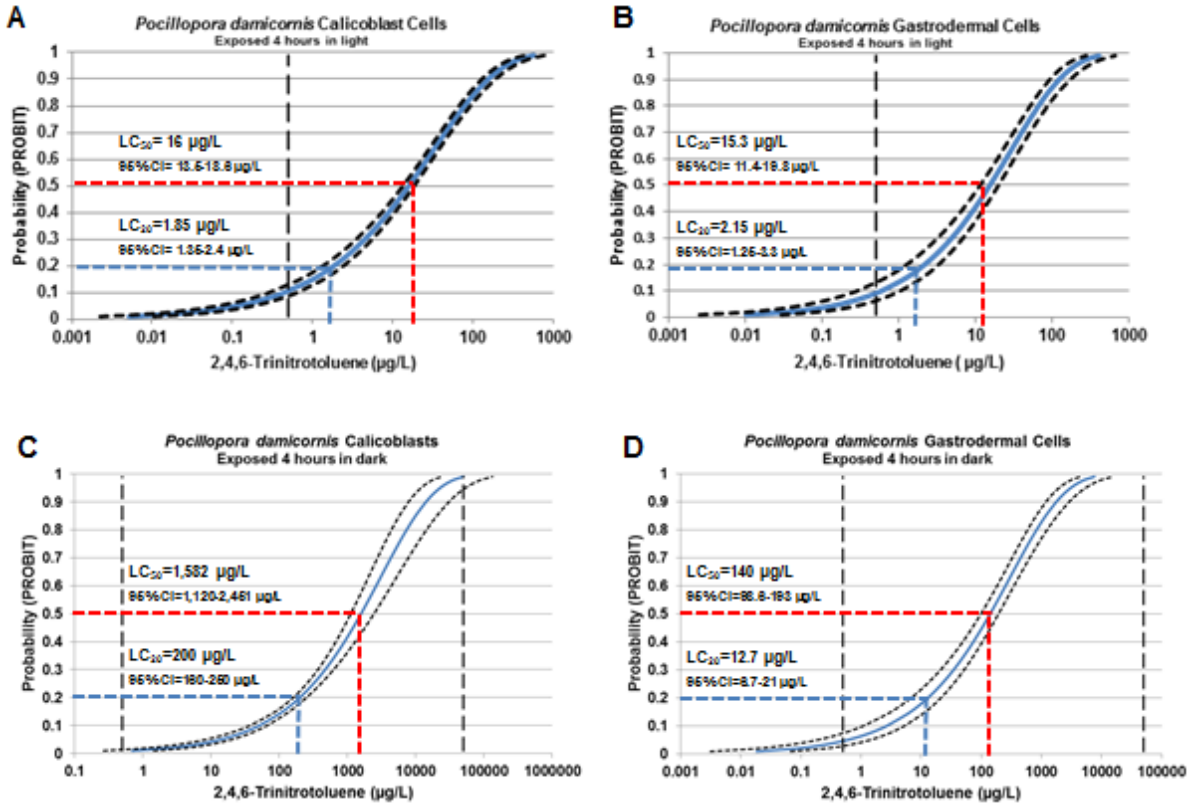


Figure 20. Results of PROBIT analysis for LC_{50} and LC_{20} values for *Pocillopora damicornis* coral cells exposed to various concentrations of TNT in light and dark conditions. A) *P. damicornis* calicoblast cells in light conditions. B) *P. damicornis* gastrodermal cells exposed in light conditions. C) *P. damicornis* calicoblast cells exposed in darkness. D) *P. damicornis* gastrodermal cells exposed in darkness. Vertical bars indicate dose range tested.

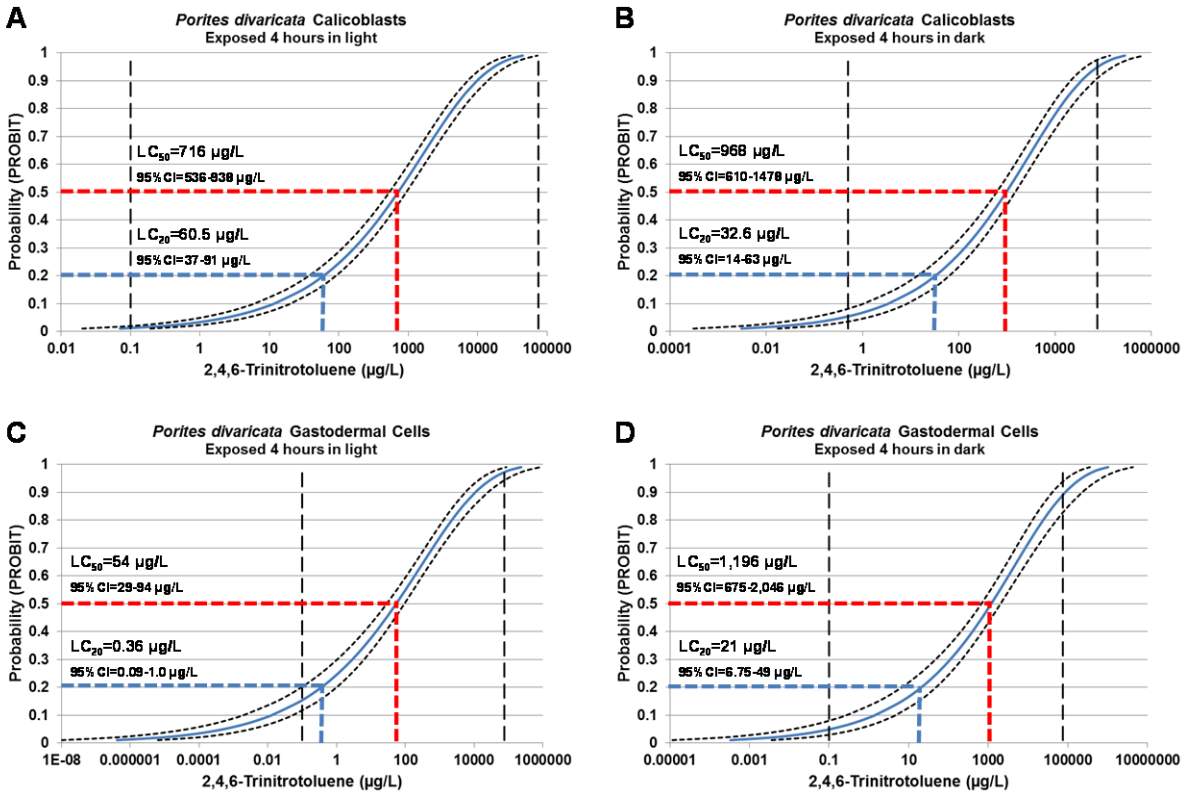


Figure 21. Results of PROBIT analyses for LC_{50} and LC_{20} values for *Porites divaracata* coral cells exposed to TNT in light and dark conditions. A) *P. divaricata* calicoblast cells in light. B) *P. divaricata* calicoblast cells in dark. C) *P. divaricata* gastrodermal cells in light. D) *P. divaricata* gastrodermal cells in dark. Vertical bars indicate dose range tested.

Symbiodinium sp. Clade B Culture Toxicity Testing

Symbiodinium Clade B was exposed to a geometric dose range (ratio=2) of TNT from 250 µg/L to 8,000 µg/L. Cell growth was monitored as one endpoint. No significant difference was found between the carrier control and media control. Thus the data were evaluated as percent of carrier control at 96 h. Significant effects were determined by a one-way ANOVA with the post-hoc Dunnett's multiple comparison for treatments versus carrier control. The NOEC was <250 µg/L and the LOEC was 250 µg/L (Fig. 22A; Table 8). A modified PROBIT using a non-linear regression analysis estimated the EC_{50} as 2,910 µg/L.

Photosynthetic efficiency of the *Symbiodinium* was used as a measure of the health condition of the photosystems. Effective quantum yield (Y(II)) was used to calculate estimated EC_{50} values which were estimated as 2,690 µg/L using a non-linear regression. This parameter mirrored the cell count data. Significant effects were determined by a one-way ANOVA with the post-hoc Dunnett's multiple comparison test for treatments versus carrier control. The NOEC was <250 µg/L and the LOEC was 2,500 µg/L (Fig. 22B; Table 8).

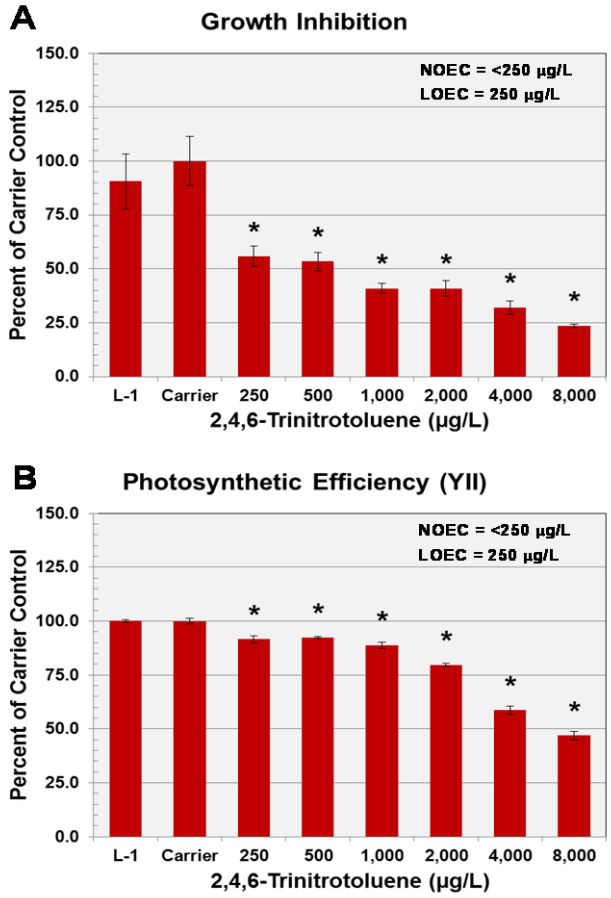


Figure 22. *Symbiodinium* Clade B cultures exposed to various concentrations of TNT for 96 h. Bars show treatment means with ±1 SEM. Treatment means with * differed significantly at α = 0.05, one-way ANOVA followed by Dunnett's test for multiple comparisons versus the carrier control. A) Growth inhibition as determined by direct cell counts. B) Photosynthetic efficiency (Y(II)) measured using an Imaging-PAM fluorometer. NOEC = no-observed-effect concentration. LOEC = lowest-observed-effect concentration.

Biotic and abiotic controls along with initial, 48 h and 96 h exposure media were analyzed by LC Mass spectroscopy by EPA method 3535A to determine concentrations and to evaluate the behavior of these compounds under the experimental conditions. Results indicated that over 99% of the initial TNT was not detected at 96 h except for the two highest concentrations (8,000 µg/L and 4,000 µg/L) which were approximately 0.15-0.2% of the initial concentrations. Two metabolites (4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene) were consistently measured at 96 h and accounted for approximately 13% on average of the initial TNT concentrations. The abiotic controls showed similar concentrations to the initial target concentrations indicating that the physical (i.e., lighting, temperature, test media) experimental conditions did not contribute to the loss of the compound in the biotic test samples found at the end of the experiment. Due to the

breakdown of TNT in a static 96 h experiment, future testing with this compound would require media changes at least every 8-12 hr.

Coral Fragment Exposure Experiments

Visual Condition Scoring

Porites divaricata were fragmented and cultured for at least 30 days prior to the TNT exposure experiment. The experimental design involved nine replicated treatments with treatment concentrations of 5, 25, 100, 500, 2,500, 12,500, 25,000 µg/L, plus controls. Corals were visually scored at 96 h for signs of adverse health effects using a set of characters recognized as associated with adverse health condition. These included tentacle retraction, polyp retraction, perforated retraction (coenenchyme tissue retracting from the skeleton) and necrosis. Mild polyp

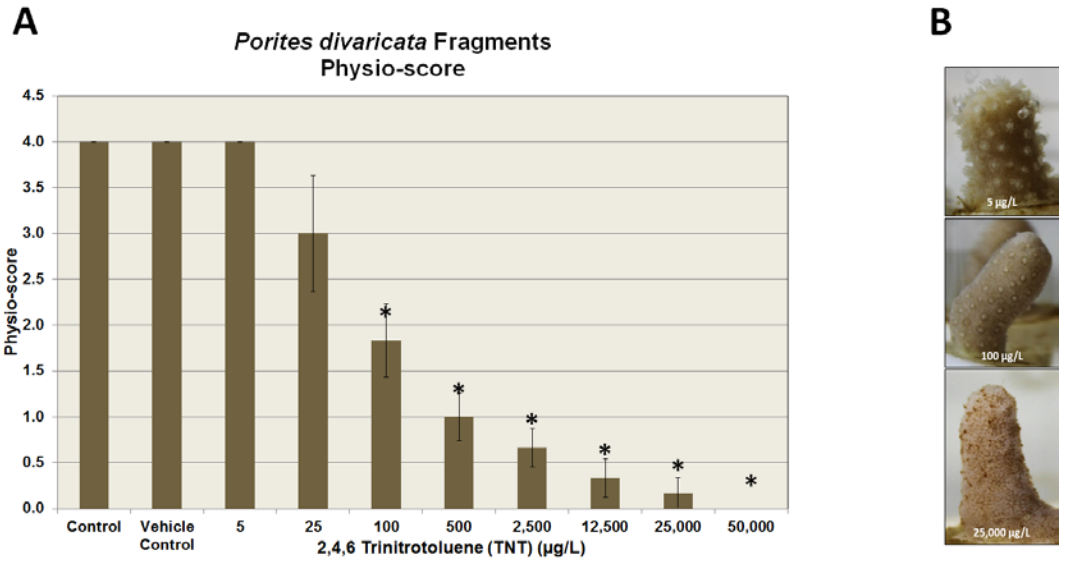


Figure 23. Physio-scoring of *Porites divaricata* fragments exposed to various concentrations of TNT at 96 h for all parameters combined. A) Scoring is based on visual observations of the coral's response to the exposure conditions. Four criteria were scored tentacle behavior, polyp behavior, tissue condition and general condition. Scoring as follows: tentacle extension=1, tentacle retraction=0; polyp extension=1, polyp retraction=0; intact tissue=1, tissue perforation and retraction=0; healthy tissue=1, necrosis=0. Treatment means with * differed significantly at $\alpha = 0.05$, one-way ANOVA followed by Dunnett's test for multiple comparisons versus the carrier control. B) Accompanying photographs of the appearance of *P.div* fragments at 96 h of exposure to various concentrations of TNT. *Photos by Craig Downs.*

retraction was noted in some fragments exposed to 25 µg/L (25 ppb) TNT with signs of an increasing adverse condition over the remaining concentrations. Significant tissue loss was observed at concentrations above 12,500 µg/L (12.5 ppm). Based on these scoring criteria, fragments were scored 0 or 1 for these four characteristics. A score derived by combining scores from each parameter is shown in Fig. 23A. When scoring criteria were subjected to statistical analysis individually, using an ANOVA with Dunnett's Method, significant ($p<0.001$) effects were seen with tentacle retraction at exposure concentrations of 500 µg/L (500 ppb) and greater.

Polyp retraction was significant ($p<0.05$) at 100 µg/L (100 ppb) and greater, while tissue perforation and retraction was evident at concentrations of 500 µg/L and greater. The most severe condition, necrosis, indicating extensive tissue loss from the skeleton was significant ($p<0.05$) at 25,000 µg/L (25 ppm) and greater.

Histopathology

Fragments exposed to various concentrations of TNT for 96 h were examined for histopathological changes in tissues. Control fragments were sampled at the termination of the experiment at 96 h for comparison. Tissues from the control samples and lowest dosed (5 µg/L) presented with normal histological features (Fig. 24). The epidermis exhibited cuboidal to short columnar epithelial cells; zooxanthellae were intact and normally situated in the gastrodermis. The calicodermis appeared active with extensions and thin secretions of organic matrix used for attachment to the skeleton. Mesoglea was thick and muscle fibers were well-formed. There were abundant nematocysts and spirocysts in the tentacles. Nematocysts and acidophilic granular

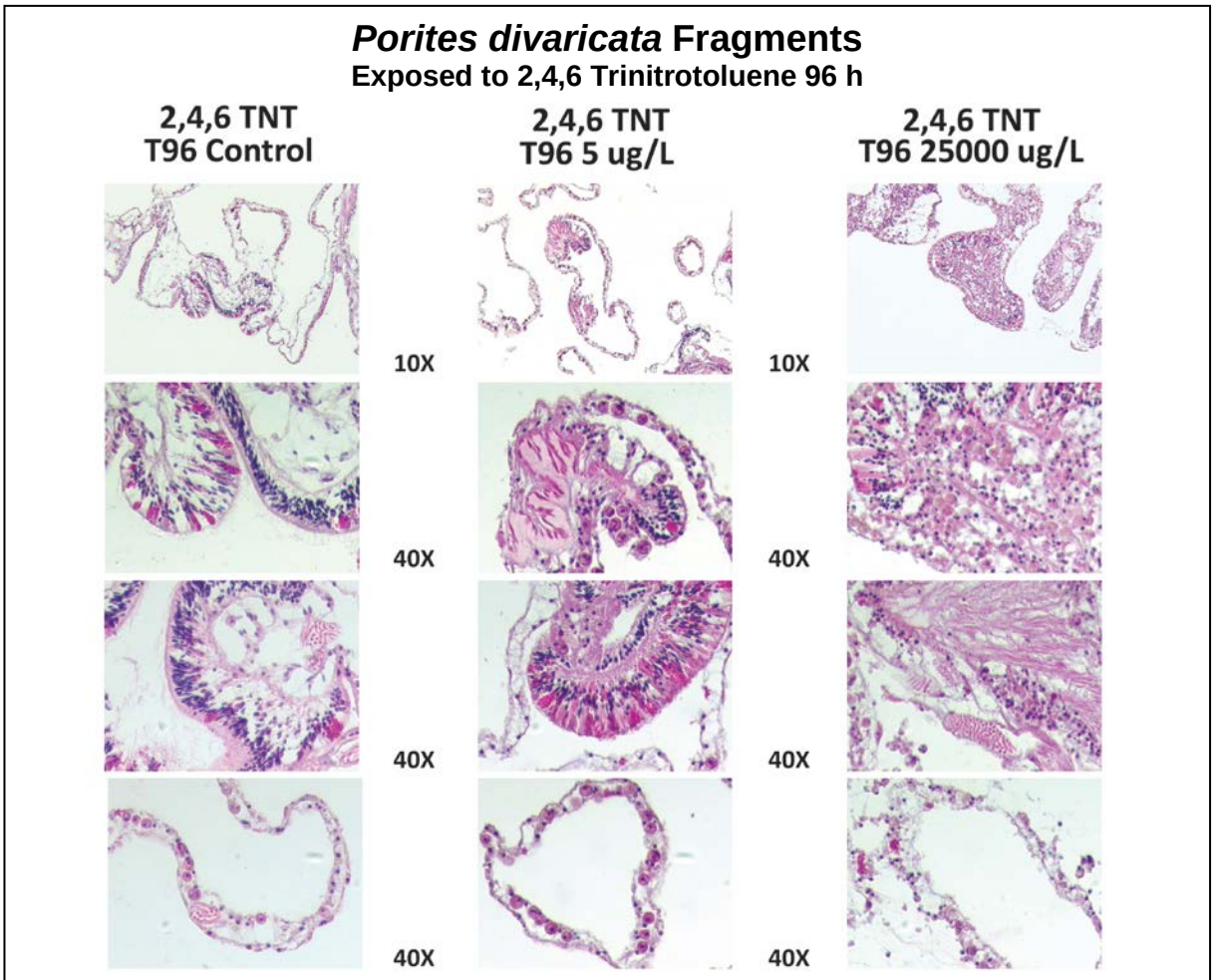


Figure 24. Histopathology of *Porites divaricata* control, low dose (5 µg/L) and high dose (25,000 µg/L) TNT exposures for 96 h. Photomicrographs of hematoxylin and eosin stained tissues show intact healthy tissues in the control (left panel) and the lowest concentration (middle panel); however, the 25,000 µg/L treated specimens displays moderate to severe necrosis, dissociated tissue and debris. *Photomicrographs by Athena R. Burnett.*

gland cells were also present in the cnidoglandular bands of mesenterial filaments. At 96 h, the 25,000 µg/L TNT treatment displayed moderate to severe necrosis and extensive dissociated tissue.

Furthermore, in the high-dosed tissues, gastrodermal cells have pyknotic nuclei and nuclei that are pushed to the cell perimeter creating a crescent moon shape (Fig. 25). Some gastrodermal cells appear swollen and many zooxanthellae are lysing. The calicodermis appears attenuated with reduction in extension of fibers that infilter the skeleton. Some areas of the calicodermis also show marked attenuation and some cell loss. The gastrodermis was also attenuated in the polyps and where the coenenchyme was intact. Muscle fibers were reduced with the mesoglea appearing more fibrous.

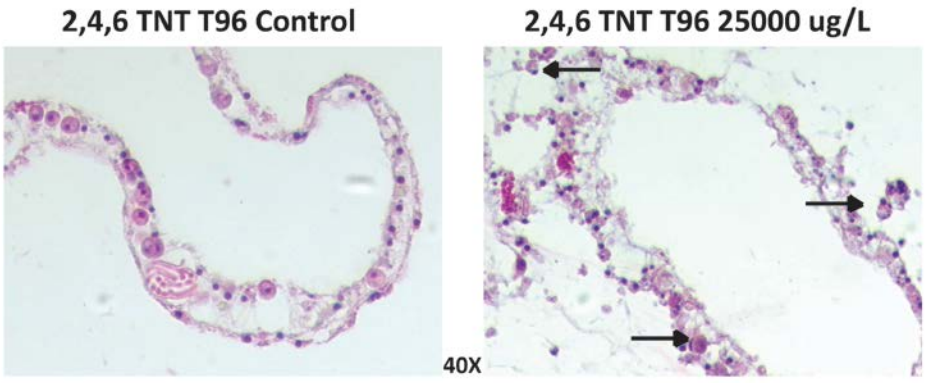


Figure 25. Close-up photomicrographs of *Porites divaricata* control and 25,000 µg/L TNT 96 h treatment. Photomicrographs of hematoxylin and eosin stained tissues show intact healthy tissues in the control (left panel) while the 25,000 µg/L treated specimens shows gastrodermal cells with pyknotic nuclei (arrows), lysing zooxanthellae, dissociated tissue, and debris. *Photomicrographs by Athena R. Burnett.*

Transmission Electron Microscopy (TEM)

Intact fragments of *Porites divaricata* were exposed to various concentrations of TNT and sampled after 16 h and 96 h incubation for histology and TEM. Changes in cellular ultrastructure could be detected after 16 h of dark exposure, as illustrated in Fig. 26. Control samples in Fig. 26A show normal ultrastructure of two gastrodermal cells, each containing a zooxanthella. At the intermediate concentration (2,500 µg/L), gastrodermal cells show increased vacuolization around three zooxanthellae and vacuole formation in adjacent cells. The lower magnification view (Fig. 26C) of the 25,000 µg/L treatment shows generalized cellular pathology with disruption of cellular ultrastructure. Notable is the vacuolization of gastrodermal cells around their zooxanthella, disruption of zooxanthella ultrastructure and mitochondrial swelling.

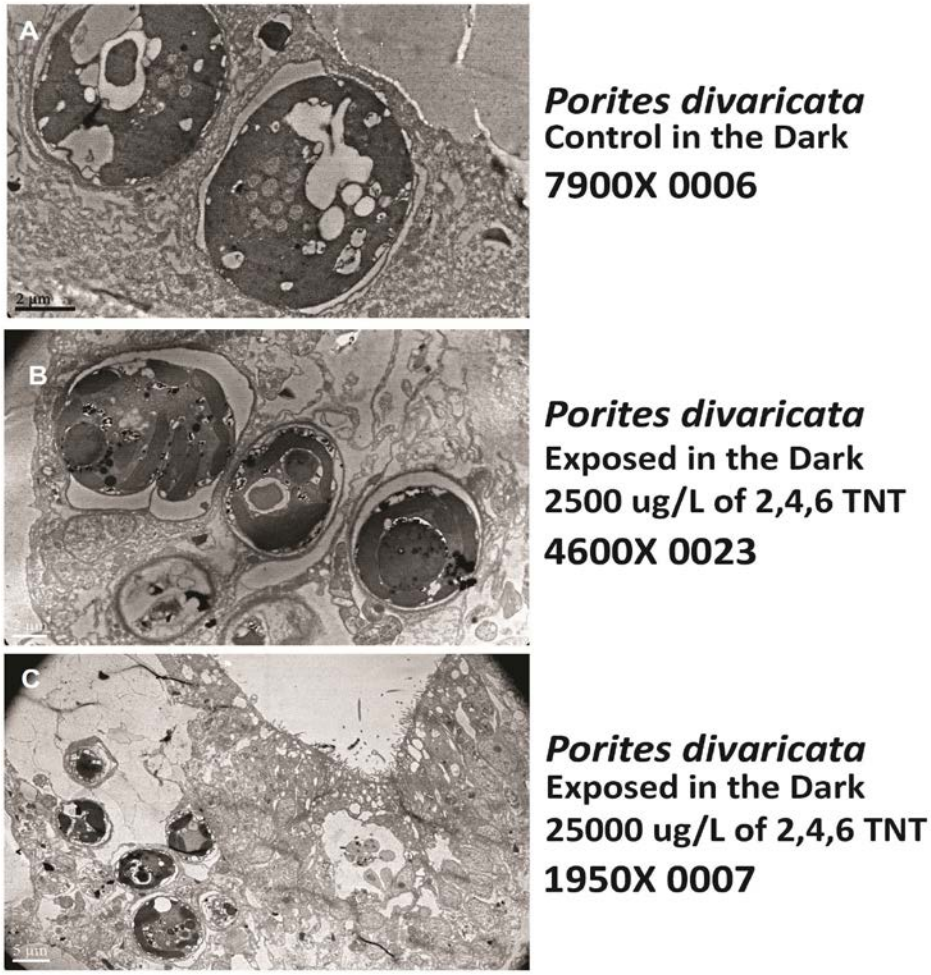


Figure 26. Transmission electron micrographs of *Porites divaricata* fragments exposed to various concentrations of TNT for 16 h in darkness. A) Control containing two zooxanthellae situated in gastrodermal cells with normal ultrastructure. B) Intermediate TNT treatment (2,500 µg/L) shows increased vacuolization around zooxanthellae. C) Lower magnification view of gastrodermal cells exposed to 25,000 µg/L TNT treatment shows generalized cellular pathology characterized by disruption of cellular ultrastructure. Notable is the vacuolization of gastrodermal cells, disruption of zooxanthellae ultrastructure and mitochondrial swelling.

Cellular Physiological Responses

Two cellular physiological (biomarker) assays were conducted on tissues from three time points [16 h (dark only), 24 h (16 h dark:8 h light) and 96 h (16:8 dark:light cycles)] of *Porites divaricata* coral fragments exposed to various concentrations of TNT. Tests for significant differences between treatment groups and controls were performed using a one-way ANOVA.

Total porphyrin levels in tissues of *P. divaricata* fragments exposed to varying concentrations of TNT following 16 h of darkness showed no significant difference in total porphyrins ($\alpha = 0.05$, one-way ANOVA followed by Dunnett's post-hoc test) from controls. The NOEC estimate for *P. divaricata* exposed to TNT in the dark for 16 h is >25,000 µg/L (25 ppm).

However after 16 h dark plus 8 h of light (24 h time point), porphyrin levels were significantly reduced at concentrations greater than 2,500 µg/L ($\alpha = 0.05$, Dunnett's Method; $p < 0.006$). The NOEC estimate for *P. divaricata* exposed to TNT for 24 h is 500 µg/L (500 ppb). The LOEC estimate is 2,500 µg/L (2.5 ppm) (Fig. 27A). The data failed to generate valid PROBIT models for the 24 h samples, because the levels did not reach the threshold of 50% change in the effects level; thus effects concentrations (EC) could not be determined.

After 96 h (diurnal cycles of 16 h dark: 8h light), porphyrin levels were significantly reduced at concentrations greater than 2,500 µg/L ($\alpha = 0.05$, one-way ANOVA followed by Dunnett's post-hoc test; $p<0.001$). The NOEC estimate for *P. divaricata* exposed to TNT for 96 h was 500 µg/L (500 ppb). The LOEC estimate was 2,500 µg/L (2.5 ppm) (Fig. 27B). A three parameter non-linear logistic regression was used to estimate the EC_{50} value of 5,416 µg/L (5.4 ppm) for porphyrin levels at 96 h of TNT exposure (Table 8).

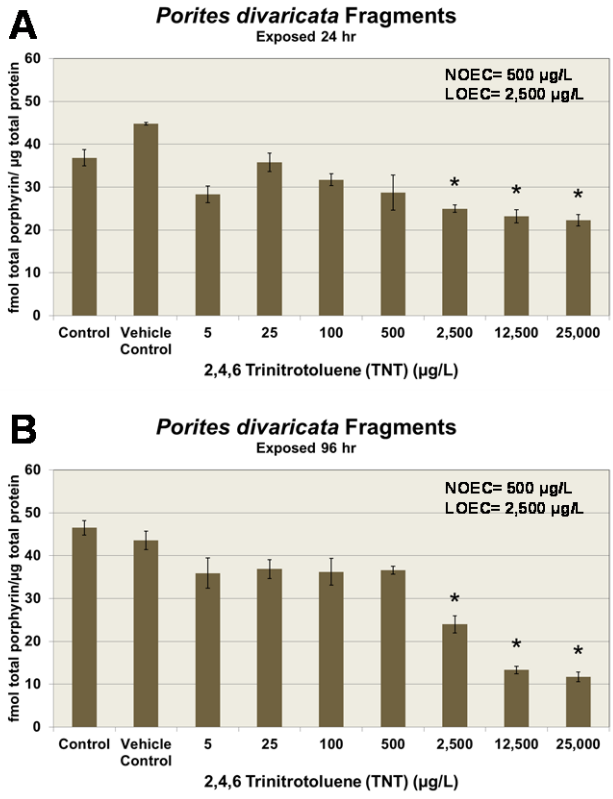


Figure 27. Total porphyrin levels in *Porites divaricata* fragments exposed to various concentrations of TNT. Bars show treatment means with ±1 SEM. Treatment means with * differed significantly at $\alpha = 0.05$, one-way ANOVA followed by Dunnett's test for multiple comparisons versus the carrier control. A) *P. diviaricata* fragments 24 h exposure. B) *P. divaracata* fragments 96 h exposure. NOEC=no-observed-effect concentration. LOEC=lowest-observed-effect concentration.

These data indicate that TNT can depress the porphyrin metabolic pathway and the depression appears to be exacerbated with light exposures. With time (96 h), depression of porphyrin levels were further depressed at 12,500 µg/L (12.5 ppm). No further depression in total porphyrins was detected at 25,000 µg/L (25 ppm).

No significant differences in the level of DNA AP site numbers were found at any of the time points or concentrations analyzed. There was high variability among all of the samples including the control. The 16 h time point samples showed no significant differences from control values. The 24 and 96 h time points however had over 50% of the samples that were greater than the standard curve and required re-analysis (Fig. 28).

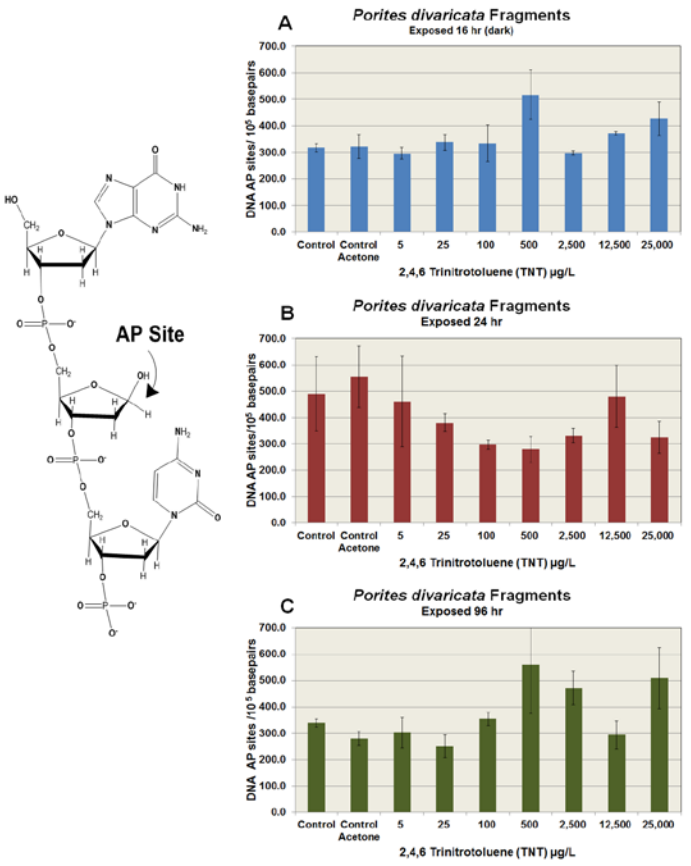


Figure 28. Number of DNA apurinic/apyrimidinic lesions in *Porites divaricata* fragments exposed to various concentrations of TNT. Bars show treatment means with ±1 SEM. A) *Pdiv* fragments 16 h (dark) exposure. B) *Pdiv* fragments 24 h exposure. C) *Pdiv* fragments 96 h exposure. No statistically significant differences were found between treatments.

The nature of this high variability of DNA AP site quantification is unclear. From the literature TNT is expected to create DNA damage, however, it is not known whether the DNA damage creates these particular lesions or affects the BER (base excision repair) pathway, which is responsible for repairing these lesions. There is also evidence that TNT adducts to DNA (and protein). One hypothesis for the high variability is that the TNT exposure may affect the binding of the Aldehyde Reactive Probe (ARP) used in this assay to bind DNA for quantification of abasic sites.

These results prompted an investigation into alternative assays to measure DNA damaging effects of genotoxic compounds such as TNT. In response to the failure of the DNA AP assay to detect damage in these laboratory exposure samples, HEL has developed a double-stranded DNA assay that can accommodate both cultured cells and whole corals. The technology is similar to the COMET assays in that it measures "tails" of double stranded or single-stranded DNA breaks. It is different from the COMET assay in that it is quantitative, using standards. This technology is being tested in both field samples and laboratory dosed samples.

Simultaneously, the NOAA laboratory has developed a DNA mutation screening assay to coral mtDNA for mutations from genotoxic chemical exposure. The details of that assay are provided in the Technical Approach section of this report and in Appendix III.

Summary of Toxicity Testing of 2,4,6-Trinitrotoluene

Summary reference toxicity values are presented in the following two tables. Table 7 provides summary information for NOEC and LOEC values that were statistically significantly ($P<0.05$) different from control treatments. Table 8 provides summary information for effect concentration values for either lethality (LC) or sub-lethal effects (EC) for all testing of coral for TNT.

Table 7. Summary of no-observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) for all experiments exposing coral specimens to TNT.

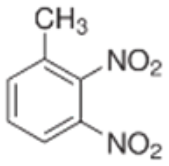
Test Organism	NOEC 2,4,6 Trinitrotoluene	LOEC 2,4,6 Trinitrotoluene
Porites divaricata Calicoblast Cells – dark 4 h	100 µg/L	500 µg/L
Porites divaricata Calicoblast Cells – light 4 h	100 µg/L	500 µg/L
Porites divaricata Gastrodermal Cells – dark 4 h	25 µg/L	100 µg/L
Porites divaricata Gastrodermal Cells – light 4 h	25 µg/L	100 µg/L
Pocillopora damicornis Calicoblast Cells – light 4 h	<0.5 µg/L	0.5 µg/L
Pocillopora damicornis Calicoblast Cells – dark 4 h	25 µg/L	100 µg/L
Pocillopora damicornis Gastrodermal Cells – light 4 h	0.5 µg/L	5 µg/L
Pocillopora damicornis Gastrodermal Cells – dark 4 h	0.5 µg/L	5 µg/L
Symbiodinium Clade B from *Pocillopora damicornis* – 96 h Growth Inhibition	<250 µg/L	250 µg/L
Symbiodinium Clade B from *Pocillopora damicornis* – 96 h Photosynthetic Efficiency (YII)	<250 µg/L	250 µg/L
Porites divaricata Fragment Total Porphyrin – 96 h	500 µg/L	2,500 µg/L

Table 8. Summary of lethal concentrations ($LC_{50,}$ LC_{20}) and effect concentrations (EC_{50}, EC_{20}) for all experiments exposing coral specimens to TNT.

Test Organism	LC_{50} 2,4,6 Trinitrotoluene	LC_{20} 2,4,6 Trinitrotoluene
Porites divaricata Calicoblast Cells – dark 4 hr	968 µg/L 95% CI = 610-1478 µg/L	32.6 µg/L 95% CI = 14-63 µg/L
Porites divaricata Calicoblast Cells – light 4 hr	716 µg/L 95% CI = 536-938 µg/L	60.5 µg/L 95% CI = 37-91 µg/L
Porites divaricata Gastrodermal Cells – dark 4 hr	1196 µg/L 95% CI = 675-2046 µg/L	21 µg/L 95% CI = 6.75-49 µg/L
Porites divaricata Gastrodermal Cells – light 4 hr	54 µg/L 95% CI = 29-94 µg/L	0.36 µg/L 95% CI = 0.09-1 µg/L
Pocillopora damicornis Calicoblast Cells – light 4 hr	16 µg/L 95% CI = 13.6-18.8 µg/L	1.85 µg/L 95% CI = 1.35-2.4 µg/L
Pocillopora damicornis Calicoblast Cells – dark 4 hr	1582 µg/L 95% CI = 1120-2451 µg/L	200 µg/L 95% CI = 160-250 µg/L
Pocillopora damicornis Gastrodermal Cells – light 4 hr	15.3 µg/L 95% CI = 11.4-19.8 µg/L	2.15 µg/L 95% CI = 1.26-3.3 µg/L
Pocillopora damicornis Gastrodermal Cells – dark 4 hr	140 µg/L 95% CI = 98.6-193 µg/L	12.7 µg/L 95% CI = 6.7-21 µg/L
	EC_{50}	EC_{20}
Symbiodinium Clade B from *Pocillopora damicornis* – 96 hr Growth Inhibition	544 µg/L 95% CI = 261-1134 µg/L	17 µg/L 95% CI = 2.67-108 µg/L
Symbiodinium Clade B - 96 hr From *Pocillopora damicornis* Photosynthetic Efficiency (YII)	7039 µg/L 95% CI = 6118-8098 µg/L	1908 µg/L 95% CI = 1498-2431 µg/L
Porites divaricata Fragment Total Porphyrin	5416 µg/L 95% CI = 2121-8712 µg/L	DMF

DMF= Data Model Failed

2,3-Dinitrotoluene



2,3-DNT

Background

2,3-Dinitrotoluene (CAS# 602-01-7; IUPAC, 1-methyl-2,3-dinitrobenzene; other synonyms: 2,3-DNT, MW:182.134 g/mol, color/form: yellow/crystals) is used as an intermediate in the production of toluene diisocyanate, explosives and dyes much like it's DNT counterparts. This nitroaromatic compound can be found as a soil contaminant from spills during manufacturing, landfill seepages, and leaking munitions wastes. Although it is a minor isomer (estimated at 1.54%) of Technical grade dinitrotoluene (Lent et al. 2012), it has been recognized as more toxic than its major isomers (2,4-DNT and 2,6-DNT) or the parent compound TNT, particularly for some freshwater organisms (fish, arthropods and algae). This compound has a solubility of 99 mg/L in 33.1 ppt seawater at ~ 19°C and 165 mg/L at 30°C (Prak and O'Sullivan 2007). Nipper et al. (2009) indicated a LC_{50} range of 1.8 - 12.6 µmol/L for freshwater fishes and an EC_{50} of 9.8 µmol/L for *Daphnia magna* reproduction.

Coral Cell Toxicity Testing

Coral cell toxicity assays were not conducted with 2,3-dinitrotoluene.

Symbiodinium sp. Clade B Culture Toxicity Testing

Symbiodinium Clade B was exposed for 96 h to a geometric dose range (ratio=2) of 2,3-dinitrotoluene from 160 to 5,000 µg/L. Cell growth was monitored as one of the endpoints. No significant difference was found between the carrier control and media control. Significant effects were determined by a one-way ANOVA with the post-hoc Dunnett's multiple comparison for treatments versus carrier control. The NOEC was 1,250 µg/L and the LOEC was 2,500 µg/L (Fig. 29A; Table 9). Thus the data were evaluated as percent of carrier control at 96 h. A modified PROBIT, non-linear regression analysis was used to estimate the EC_{50} value as 2,530 µg/L (Table 10).

Photosynthetic efficiency of the *Symbiodinium* was used as a measure of the health condition of the photosystems. Significant effects were determined by a one-way ANOVA with the post-hoc Dunnett's multiple comparison test for treatments versus carrier control The NOEC was <160 µg/L and the LOEC was 160 µg/L (Fig. 29B; Table 9). It is noteworthy that photosynthetic efficiency measures indicated that photosystems shutdown at a concentration of 5,000 µg/L (Fig. 29B arrow). Effective quantum yield (Y(II)) was used to calculate estimated EC_{50} values which

were estimated as 2,870 µg/L using a non-linear regression (Table 10). This parameter mirrored the cell count data.

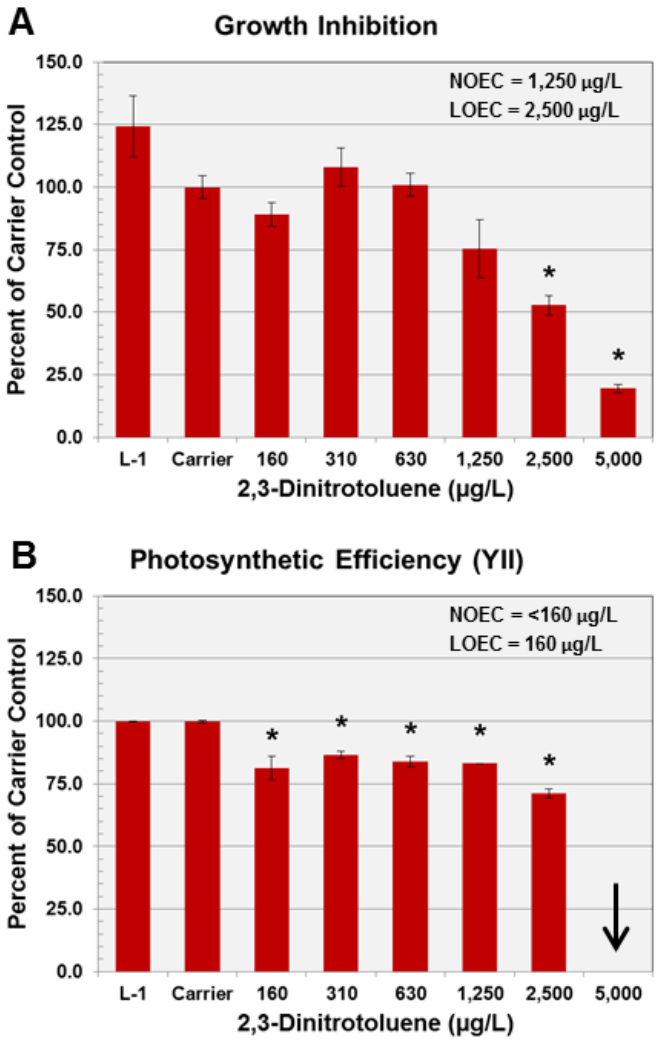


Figure 29. *Symbiodinium* Clade B cultures exposed to various concentrations of 2,3-DNT for 96 h. Bars show treatment means with ±1 SEM. Treatment means with * differed significantly at α = 0.05, one-way ANOVA followed by Dunnett's test for multiple comparisons versus the carrier control. A) Growth inhibition as determined by direct cell counts. B) Photosynthetic efficiency (Y(II)) measured using an Imaging-PAM fluorometer. Arrow indicates photosystem shut-down. NOEC = no-observed-effect concentration. LOEC = lowest-observed-effect concentration.

Coral Fragment Exposures

Pocillopora damicornis fragments were exposed to 2,3-dinitrotoluene in a time-course experimental design for 96 h. The design involved five replicated treatments with treatment concentrations of 130, 320, 800, 2,000, or 5,000 µg/L, plus carrier (acetone) control with six replicates for each treatment and four fragments in each replicate for time point sampling.

Morphology (Experiment 1)

Pocillopora damicornis fragments were initially exposed to concentrations of 2,3-dinitrotoluene ranging from 130 to 5,000 µg/L. The physiological condition of the fragments was observed every day at a prescribed time during their diurnal cycle for consistent comparisons between treatments. Observations were recorded as a physio-score (0-4, with 0=healthy and 4=severely impacted) to describe polyp behavior, tissue integrity and color. Within the initial 18 h of the experiment, corals exposed to 2,000 and 5,000 µg/L were experiencing severe tissue loss and by 36 h only an estimated 10% of tissue remained, with most of the coenenchyme (area between polyps) retracted. By the 84 h visual inspection, the 2,000 µg/L treatment had no tissue remaining on the skeleton, while the 5,000 µg/L treatment had a few remaining remnants of tissue in the corallite (skeletal support for individual polyps). By 60 h, coral fragments exposed to 800 µg/L were experiencing tissue color paling and polyps fully retracted. Treatments of 130 and 320 µg/L exhibited

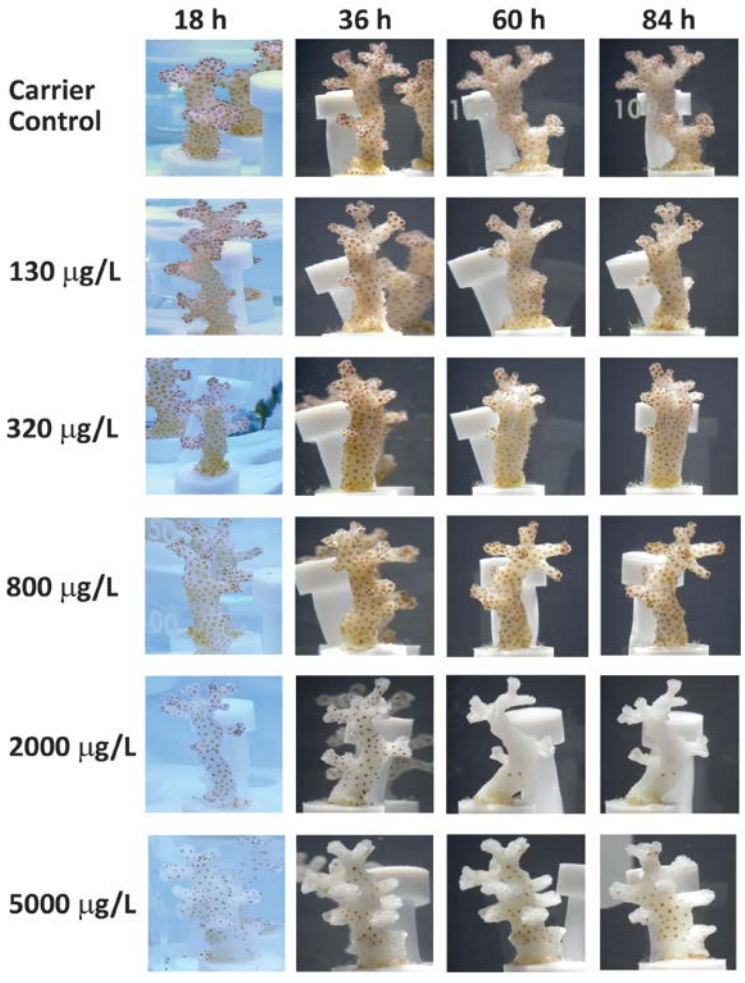


Figure 30. Photographs of *Pocillopora damicornis* fragments exposed to various treatments of 2,3-DNT for 96 h. Photographs depict visual inspections of the fragment's tissue integrity, color and polyp behavior. *Photos by Athena Burnett.*

complete polyp retraction by 36 h and remained in this condition for the duration of the experiment. The carrier (acetone) control exhibited normal behavior throughout the experiment (Fig. 30).

Histopathology (Experiment 1)

Fragments exposed to various concentrations of 2,3-DNT for 48 h and 96 h were examined for histopathological changes in tissues. Time zero (T0) fragments were sampled at the outset of initiating the experiment for comparison. Tissues from the T0 samples presented with normal histological features (Fig. 31). The epidermis exhibited cuboidal to short columnar epithelial cells; zooxanthellae were intact and normally situated in the gastrodermis. The calicodermis appeared active with extensions and thin secretions of organic matrix used for attachment to skeleton. Mesoglea was thick and muscle fibers were well-formed. There were abundant

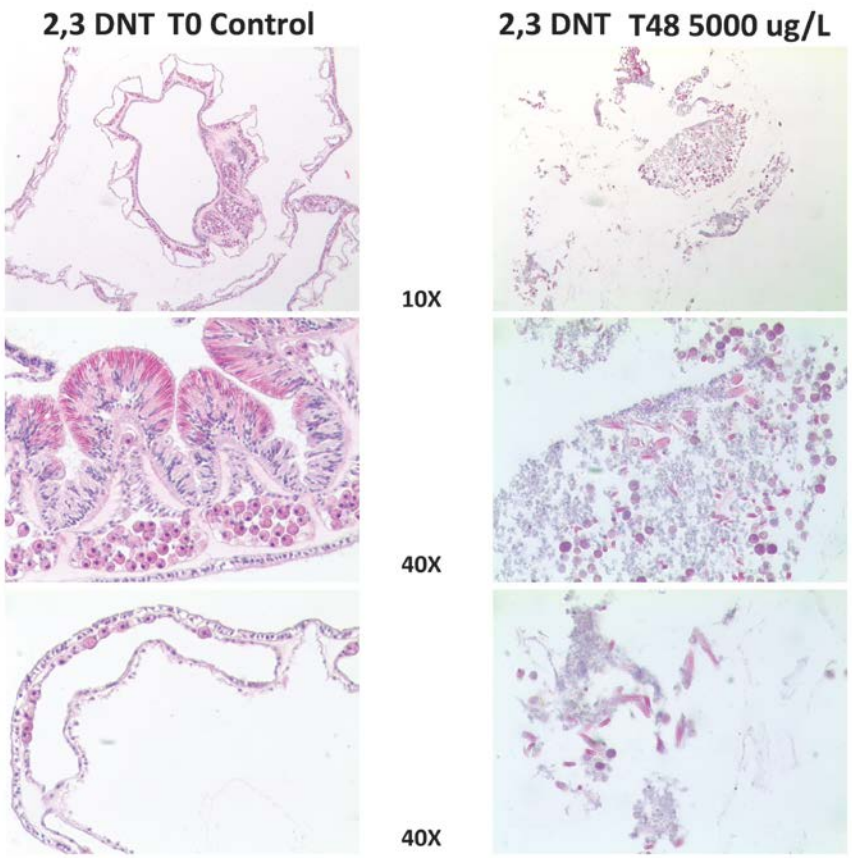


Figure 31. Histopathology of *Pocillopora damicornis* exposed to 5,000 µg/L of 2,3-DNT for 48 h. Photomicrographs of hematoxylin and eosin stained tissues show intact healthy tissues in the T0 control (left panel) while the 5,000 µg/L treated specimens displays severe necrosis, dissociated tissue, debris and necrotic zooxanthellae. *Photomicrographs by Dr. Esther Peters.*

nematocysts and spirocysts in tentacles. Nematocysts and acidophilic granular gland cells were also present in the cnidoglandular bands of mesenterial filaments. At 48 h for the highest exposure treatment, 5,000 µg/L 2,3-DNT, displayed severe necrosis and extensive dissociated tissue (Fig. 31).

The 96 h tissues displayed a gradient of histological changes from little change to massive disruption of the tissue that mirrored increases in exposure concentrations of 2,3-DNT (Fig. 32). Carrier controls showed minor changes with the epidermis becoming more columnar with scattered mucocytes, other structures were similar to the T0 samples (Fig. 31). Approximately 5-10% of the gastrodermal cells showed vacuolization with a few scattered zooxanthellae lysing.

Coral fragments subjected to the 320 µg/L treatment showed marked attenuation of the epidermis, with cuboidal epithelium, with pyknotic nuclei and an increase of mucocytes or vacuolated cells, including the tentacles. Gastrodermal cells had normal nuclei, though some appear swollen with a few zooxanthellae lysing. However, there was a moderate loss of zooxanthellae on the oral disk and the tentacle gastrodermis, with some lysing. The gastrodermis was also attenuated in the polyps and coenenchyme. The calicodermis had moderate to marked attenuation and reduced extensions; however, the mesenterial filaments and tentacles were still intact. The mesoglea appeared swollen and displayed some reduction in muscle fibers.

Coral fragments subjected to the 800 µg/L treatment displayed severe necrosis of the epidermis with extensive vacuolization, though it remained intact for the most part as a thin layer along the surface of the mesoglea with occasional detachment in some areas. The gastrodermis appeared vacuolated or with increased mucus. The gastrodermis also appeared filled with fine eosinophilic particles or lipid droplets. Zooxanthellae were mostly lysing with large vacuoles remaining in the gastrodermal cells. Muscle fibers were reduced with the mesoglea appearing more fibrous. Calicoblasts along the mesoglea appeared missing or lysing with sloughing, vacuolated fibrous remnants were present. Single cell necrosis in the cnidoglandular bands was present as well as vacuolization in this area. Of note were cells in the calicodermis and gastrodermis that are suspected as interstitial cells containing large nuclei, prominent nucleolus with no cytoplasm visible.

The 5000 µg/L treatment had only a few remnants of necrotic cells, nematocysts, spirocysts and debris.

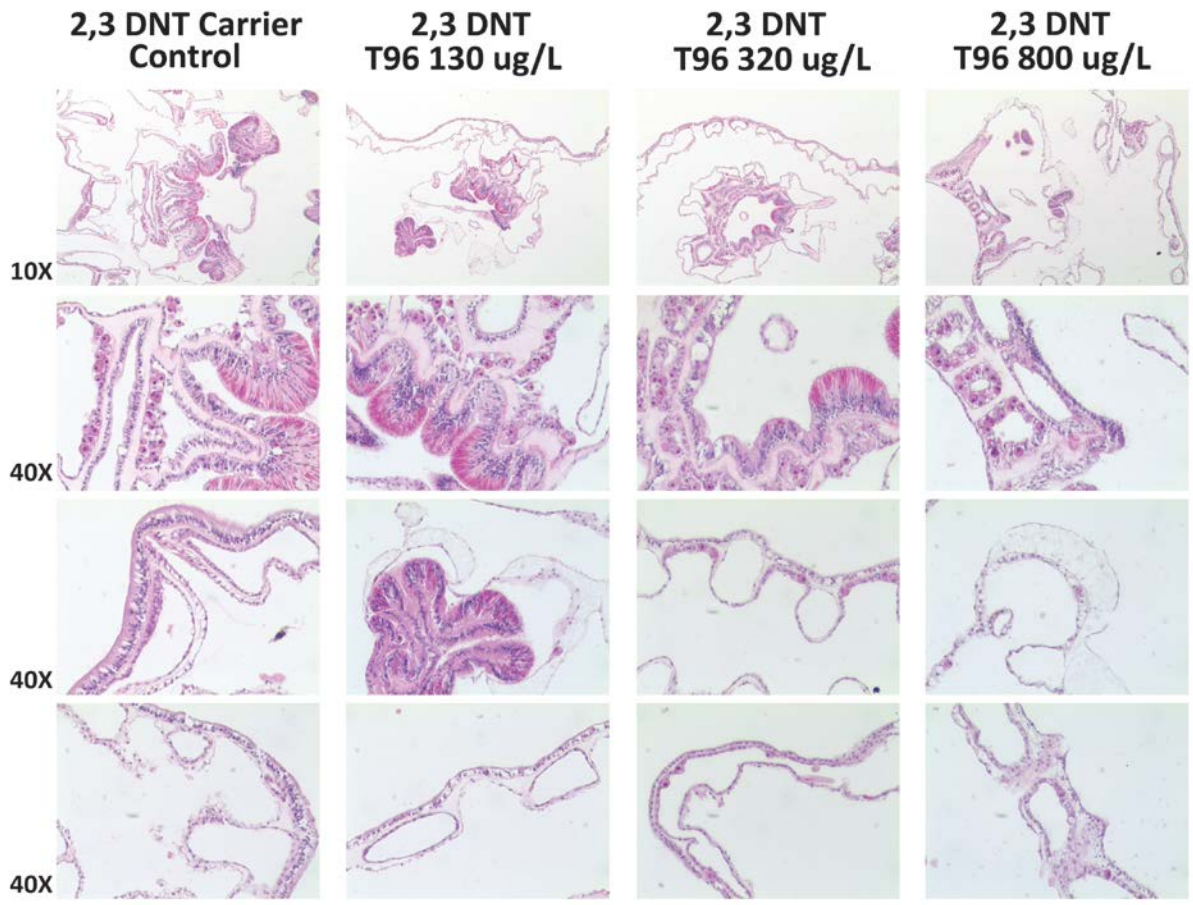


Figure 32. Histopathology of *Pocillopora damicornis* exposed to various concentrations of 2,3-DNT for 96 h. Photomicrographs are of 96 h specimens stained with hematoxylin and eosin. Tissues show a survey of the coral's epidermis, gastrodermis and calicodermis across treatments. Treatments of 2,000 and 5,000 µg/L had only tissue fragments remaining and were not included in this survey. *Photomicrographs by Dr. Esther Peters.*

Cellular Physiological Responses (Experiment 1)

Two clinical diagnostic assays were conducted on samples that remained sufficiently intact in order to gauge the response range and behavior of two cellular physiological responses of *Pocillopora damicornis* to 2,3-DNT. Total porphyrin levels were measured as an indicator of general metabolic condition. DNA AP site accumulation results from oxidative events or alkylation of DNA and is a measure of genomic integrity.

Results from experiment 1 were inconclusive. However there was noted a possible trend for porphyrin depression with increased concentration of the 2,3-DNT (Fig. 33A), while there was a trend for increased DNA AP sites (Fig. 33B). This particular cellular physiological endpoint showed a complex bi-modal behavior for a number of the munitions compounds, including 2,3-DNT. It is also worth noting that levels of AP sites were very high, and often samples exceeded the standard curve of the expected range of DNA AP sites for this particular kit, designed for mammalian diagnostics. Since another experiment was planned to capture sub-lethal exposure conditions, these samples were not re-assayed.

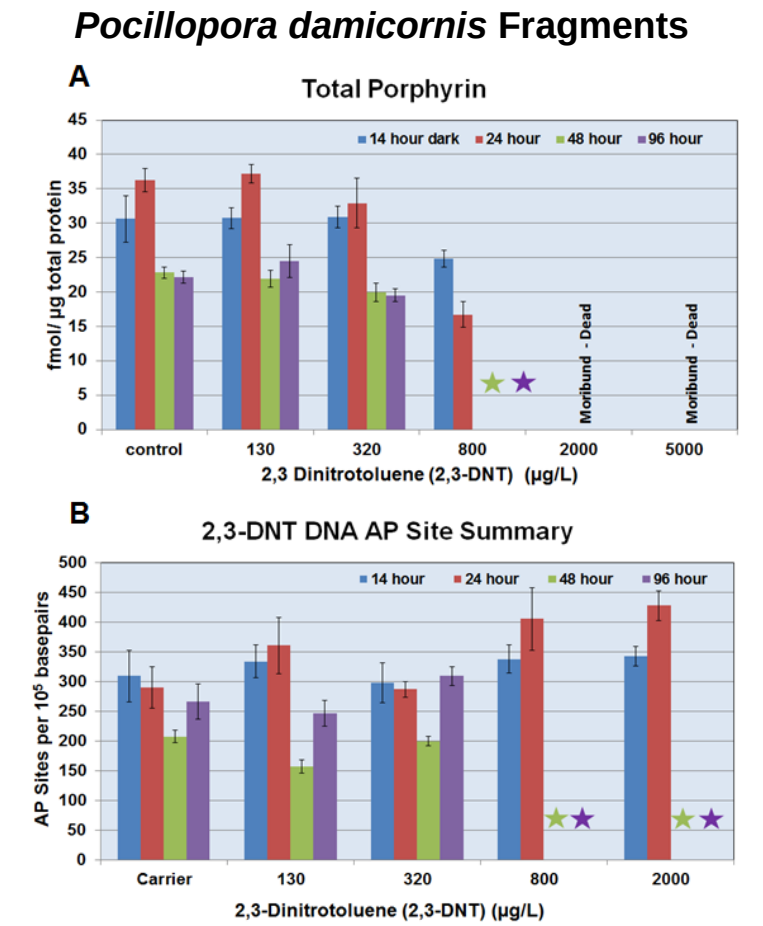


Figure 33. Porphyrin and DNA AP site analysis of tissue from *Pocillopora damicornis* fragments exposed to various concentrations of 2,3-DNT. Bars show treatment means with ±1 SEM. A) Total porphyin levels summarized across all treatments and all time points. Stars indicate samples that were moribund or dead and thus excluded from the analyses. B) Number of DNA AP sites per 10^5 base pairs across all treatments and all time points. Stars indicate sample time points for which the tissue was not suitable for DNA extractions to perform the assay. Statistical analyses were unable to resolve any significant differences among treatments.

Sub-lethal Exposures (Experiment 2)

Pocillopora damicornis fragments were exposed to 2,3-dinitrotoluene in a time course design for 96 h. The experimental design involved treatments with concentrations of 50, 90, 162, 292 or 525 µg/L, plus carrier (acetone) control with six replicates for each treatment and four fragments in each replicate for time point sampling (repeated measures design). The new dosages were based on the initial dose range (130, 320, 800, 2,000, 5,000 µg/L) found to elicit lethality at doses above 320 µg/L at 96 h and the highest concentration caused death at 18 h visual condition scoring (Fig. 30).

Visual Condition Scoring (Experiment 2)

The physiological condition of the *Pocillopora damicornis* fragments was observed every day at a prescribed time of their diurnal cycle for most consistent comparisons between treatments. Observations were recorded as a physio-score (1-5, with 5=healthy and 1=severely impacted) to describe polyp behavior, tissue integrity and color (note this physio-score was a modification of that previously described in an attempt to capture more subtle changes). At these concentration ranges, the tissue remained intact and color remained normal in all treatments and time points. Polyp behavior did respond negatively (score=1) by fully retracting at concentrations of 162 µg/L and lower (Fig. 34).

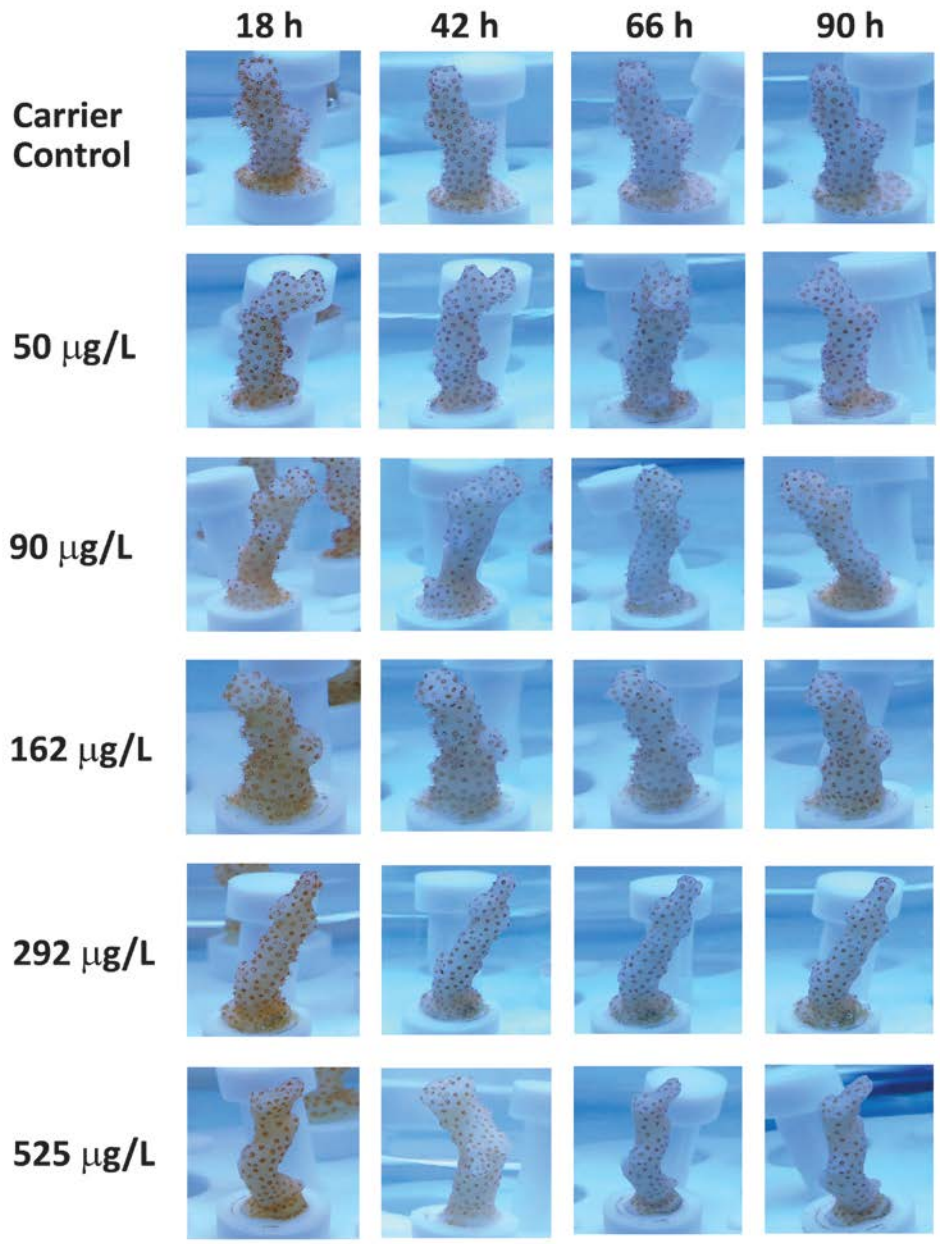


Figure 34. Photographs of *Pocillopora damicornis* fragments exposed to various treatments of 2,3-dinitrotoluene. Photographs depict visual inspections of the behavior of the fragments tissue integrity, color and polyp behavior. *Photos by Athena Burnett.*

Histopathology (Experiment 2)

Pocillopora damicornis fragments from the second exposure were collected and fixed for histological analysis but have not been analyzed.

Sub-lethal Cellular Physiological Responses (Experiment 2)

Two clinical diagnostic assays were conducted on samples to gauge the range and behavior of two cellular physiological responses of *Pocillopora damicornis* to 2,3-DNT. Total porphyrin levels were measured as an indicator of general metabolic condition. DNA AP site accumulation was also measured as an indicator of genomic integrity.

No significant ($p>0.05$) differences from control treatments were found in mean porphyrin levels of fragments at the 14, 24 and 48 h time points (Fig. 35A). At 96 h, 2,3-DNT did have a significant effect on mean porphyrin levels ($p=0.0119$) as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison test for treatments versus a control found that the two highest 2,3-DNT concentrations tested (292 and 525 µg/L) had significantly lower mean porphyrin levels ($p<0.05$) as compared to the control group (Fig. 35A,B). The NOEC was 162 µg/L and the LOEC was 292 µg/L (Table 9).

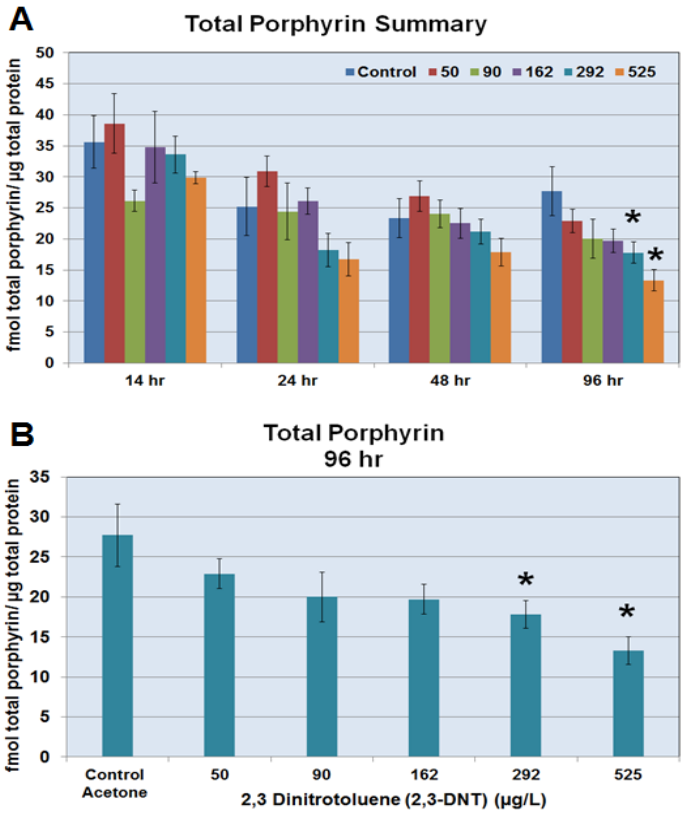


Figure 35. Porphyrin levels in tissue from *Pocillopora damicornis* fragments exposed to various concentrations of 2,3-DNT. Bars show treatment means with ±1 SEM. A) Total porphyin levels summarized across all treatments and all time points. B) Total porphyrin levels at 96 h time point. Asterisks indicate a significant effect ($p<0.0119$) as determined by a one-way ANOVA followed by Dunnett's multiple comparison test for treatments versus the acetone control.

Summary of Toxicity Testing of 2,3-Dinitrotoluene

Summary reference toxicity values are presented in the following two tables. Table 9 provides summary information for NOEC and LOEC values that were statistically significant ($p<0.05$) different from control treatments. Table 10 provides summary information for effect concentration values for either lethality (LC) or sub-lethal effects (EC) for all testing of coral for 2,3-DNT.

Table 9. Summary of no-observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) for all experiments exposing coral specimens to 2,3-DNT. ND=Not Determined.

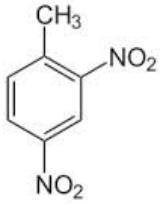
Test Organism	NOEC 2,3 Dinitrotoluene	LOEC 2,3 Dinitrotoluene
Porites divaricata Calicoblast Cells	ND	ND
Porites divaricata Gastrodermal Cells	ND	ND
Pocillopora damicornis Calicoblast Cells	ND	ND
Pocillopora damicornis Gastrodermal Cells	ND	ND
Symbiodinium Clade B – 96h *Pocillopora damicornis* Growth Inhibition	1,250 µg/L	5,000 µg/L
Symbiodinium Clade B – 96h (*Pocillopora damicornis*) Photosynthetic Efficiency (YII)	<160 µg/L	160 µg/L
Pocillopora damicornis Fragment Total Porphyrin – 96 h	162 µg/L	292 µg/L

Table 10. Summary of lethal (LC_{50}, LC_{20}) and sub-lethal effect concentrations (EC_{50}, EC_{20}) for all experiments exposing coral specimens to 2,3-DNT. ND=Not Determined.

Test Organism	LC_{50} 2,3 DIinitrotoluene	LC_{20} 2,3 Dinitrotoluene
Porites divaricata Calicoblast Cells	ND	ND
Porites divaricata Gastrodermal Cells	ND	ND
Pocillopora damicornis Calicoblast Cells	ND	ND
Pocillopora damicornis Gastrodermal Cells	ND	ND
	EC_{50}	EC_{20}
Symbiodinium Clade B – 96 h (*Pocillopora damicornis*) Growth Inhibition	2524 µg/L 95% CI = 2037-3128 µg/L	1275 µg/L 95% CI = 885-1839 µg/L
Symbiodinium Clade B- 96 h (*Pocillopora damicornis*) Photosynthetic Efficiency (YII)	2810 µg/L (Estimated)	2520 µg/L (Estimated)
Pocillopora damicornis Fragment Total Porphyrin – 96 h	DFM	DFM

DMF= Data Models Failed.

2,4-Dinitrotoluene



2,4-DNT

Background

2,4-Dinitrotoluene (CAS # 121-14-2; IUPAC, 1-methyl-2,4-dinitrobenzene; other synonyms: 2,4-DNT, Dinitrotoluol; MW: 182.134 g/mol; color/form: yellow or orange/crystals) is a major breakdown product of TNT. Its solubility is 127 mg/L at 20°C and 197 mg/L at 30°C in 33.1 ppt seawater (Prak and O'Sullivan 2006). It is used as an intermediate in the production of toluene diisocynate, polyurethanes, and dyes in the commercial and military explosives industry (e.g., waterproofing agent, plasticizer, propellant, and modifier for smokeless powders). It can be released into the aquatic environment through the improper handling and disposal of unexploded ordinance (UXO), runoff from contaminated soil and ground water from artillery ranges and manufacturing sites, and also released through aquatic warfare. Toxicity studies (Liu et al. 1983; Nipper et al. 2002; Nipper et al. 2009) indicated EC_{50} values for freshwater or marine algae of 0.1-14 µmoles/L and 96 h LC_{50} for fresh water fish or Fathead minnow of 26.3 – 32.5 mg/L and 39 mg/L NOEC and 75 mg/L LOEC for sea urchin fertilization and embryo development.

Coral Cell Toxicity Testing

Observed-effect concentrations (NOEC and LOEC)

A ten-point concentration gradient from 0.1 to 75,000 µg/L of 2,4-DNT was used for the exposures of calicoblast and gastrodermal cells from *Porites divaricata* and *Pocillopora damicornis*. Primary cells were isolated and cultured as described in the methods and plated into 24-well Teflon® plates at approximately $6.5x10^5$ to $1.0x10^6$ cells per well. Cells were exposed for 4 h in the light (295 µmol m^{-2} s^{-1}) at 25°C. Cell viability counts were performed on each well using the trypan blue exclusion dye viability assay as the endpoint for effects characterization.

Exposure of ***Porites divaricata*** **calicoblast** cells to various concentrations of 2,4-DNT showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was 0.05 µg/L (50 pptrillion) and the LOEC was 5 µg/L (5 ppb) (Fig. 36A; Table 11).

Exposure of ***Porites divaricata*** **gastrodermal** cells showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was 25 µg/L (25 ppb) and the LOEC was 100 µg/L (100 ppb) (Fig. 36B).

Exposure of ***Pocillopora damicornis*** **calicoblast** cells to various concentrations of 2,4-DNT showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison for treatments versus a control found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was 5 µg/L (5 ppb) and the LOEC was 25 µg/L (25 ppb) (Fig. 36C; Table 11).

Exposure of ***Pocillopora damicornis*** **gastrodermal** cells showed a significant effect on percent mortality ($p<0.0001$) as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison for treatments versus a control found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was 0.1 µg/L (100 pptrillion) and the LOEC was 0.5 µg/L (500 pptrillion) (Fig. 36D; Table 11).

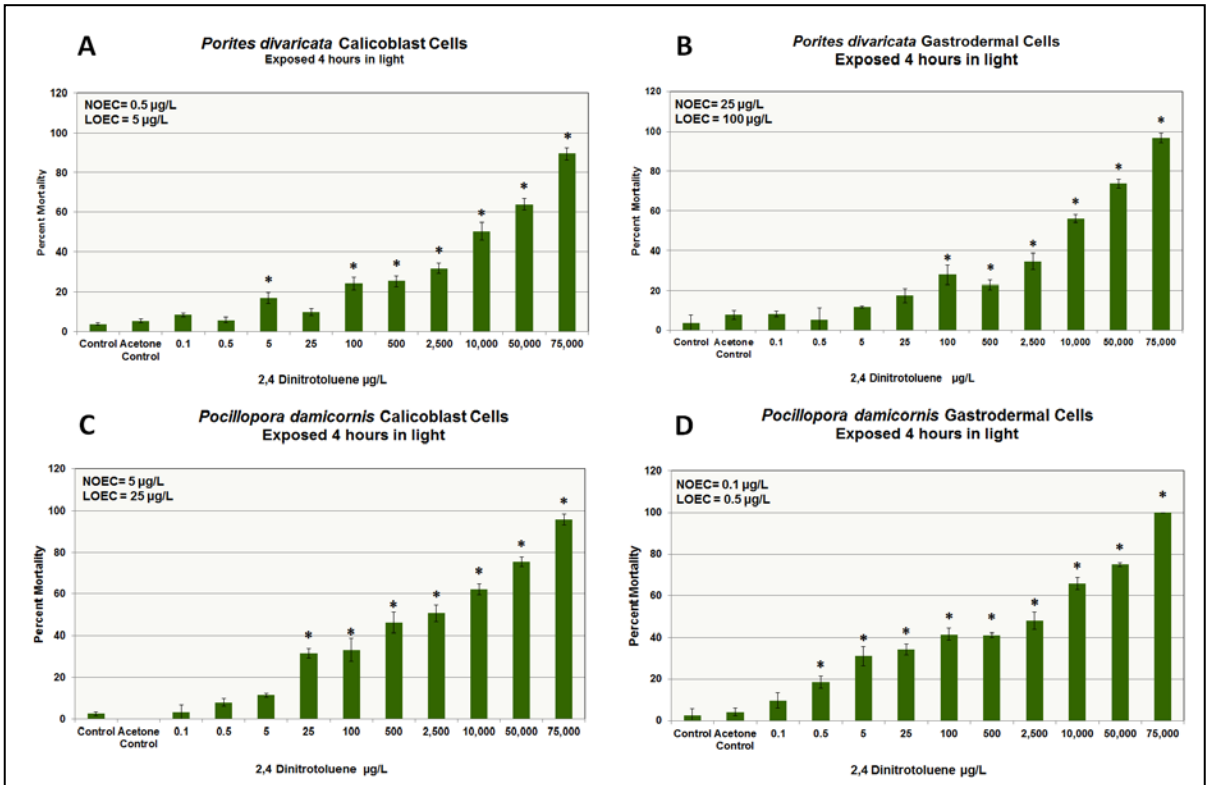


Figure 36. Percent mortality of *Porites divaricata* and *Pocillopora damicornis* cells exposed to various concentrations of 2,4-DNT. n=4 replicates per treatment. Bars show treatment means with ±1 SEM. Treatment means with * differed significantly at $\alpha = 0.05$, one-way ANOVA followed by Dunnett's test for multiple comparisons versus the carrier control. A) *P. divaricata* calicoblast cells. B) *P. divaricata* gastrodermal cell. C) *P. damicornis* calicoblast cells. D) *P. damicornis* gastrodermal cells. NOEC=no-observed-effect concentration. LOEC=lowest-observed effect concentration.

LC_{50} and LC_{20}

Porities divaricata and *Pocillopora damicornis* calicoblast and gastrodermal cell mortality data for 2,4 DNT exposures were subjected to PROBIT analysis to determine the LC_{50} and LC_{20} values and confidence intervals. The LC_{50} for ***Porites divaricata*** **calicoblast cells** was 5,818 µg/L (5.818 ppm) and the LC_{20} was 78 µg/L (78 ppb) (Fig. 37A; Table 12). The LC_{50} for ***Porites divaricata*** **gastrodermal** cells was 3,123 µg/L (3.123 ppm) and the LC_{20} was 52 µg/L (52 ppb) (Fig. 37B; Table 12). The LC_{50} for ***Pocillopora damicornis*** **calicoblast** cells was 1,284 µg/L (1.284 ppm) and the LC_{20} was 15 µg/L (15 ppb) (Fig. 37C; Table 12). The LC_{50} of ***Pocillopora damicornis*** **gastrodermal** cells was 565 µg/L (565 ppb) and the LC_{20} was 2 µg/L (2 ppb) (Fig. 37D; Table 12).

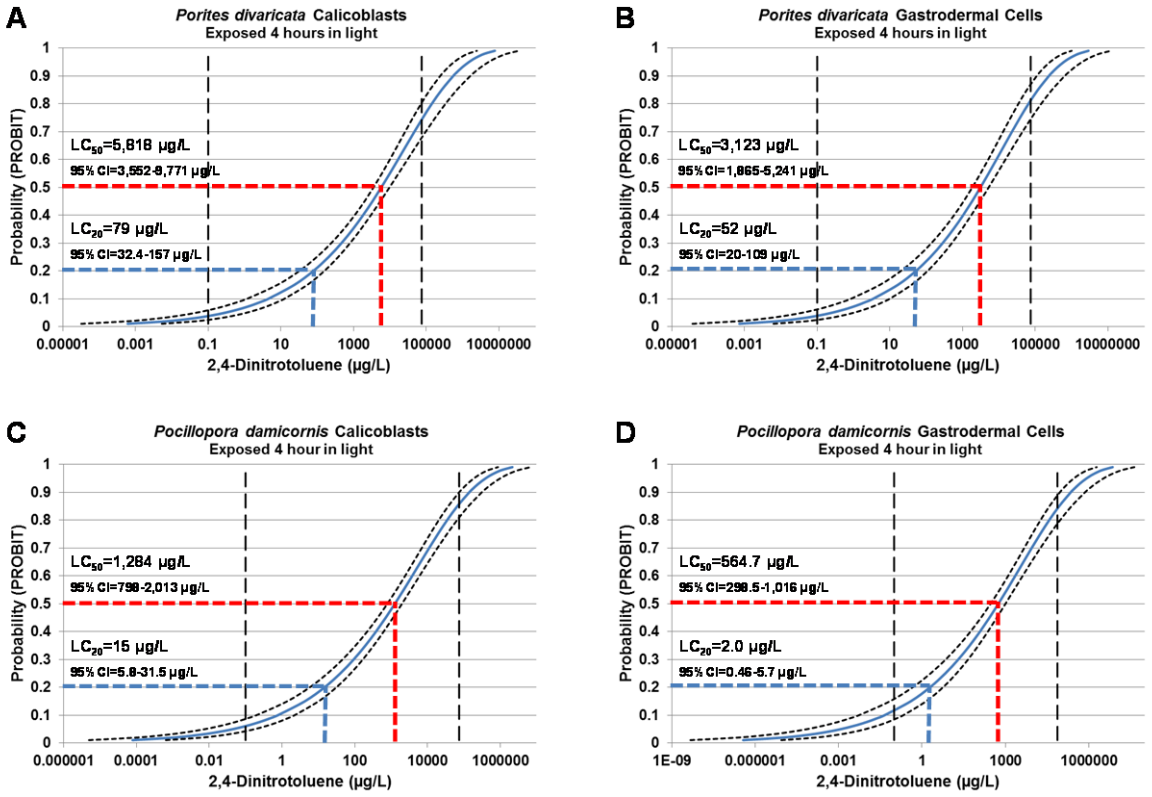


Figure 37. Results of PROBIT analyses LC_{50} and LC_{20} values for coral cells exposed to 2,4-DNT in light conditions. A) *P. divaricata* calicoblast cells. B) *P. divaricata* gastrodermal cells. C) *P. damicornis* calicoblast cells. D) *P. damicornis* gastrodermal cells. Vertical bars indicate dose range tested.

Symbiodinium sp. Clade B Culture Toxicity Testing

Symbiodinium Clade B was exposed to a geometric dose range (ratio=2) of 2,4-dinitrotoluene from 310 to 10,000 µg/L. Cell growth was monitored as one endpoint. No significant difference was found between the carrier control and media control. Thus the data were evaluated as percent of carrier control at 96 h. Significant effects were determined by a one-way ANOVA with the post-hoc Dunnett's multiple comparisons test for treatments versus carrier control. The NOEC was 310 µg/L and the LOEC was 630 µg/L (Fig. 38A). A modified PROBIT using a non-linear regression analysis of cell growth data estimated the EC_{50} as 1,315 µg/L (95% CI=880-1,964 µg/L).

Symbiodinium photosynthetic efficiency of was used as a measure of the health condition of the dinoflagellates' photosystems. Significant effects were determined by a one-way ANOVA with the post-hoc Dunnett's multiple comparison test for treatments versus carrier control. The NOEC was 630 µg/L and the LOEC was 1,250 µg/L (Fig. 38B). Effective quantum yield was used to calculate EC_{50} values using a non-linear regression which estimated the EC_{50} as 4,814 µg/L (95% CI= 4347-5331µg/L).

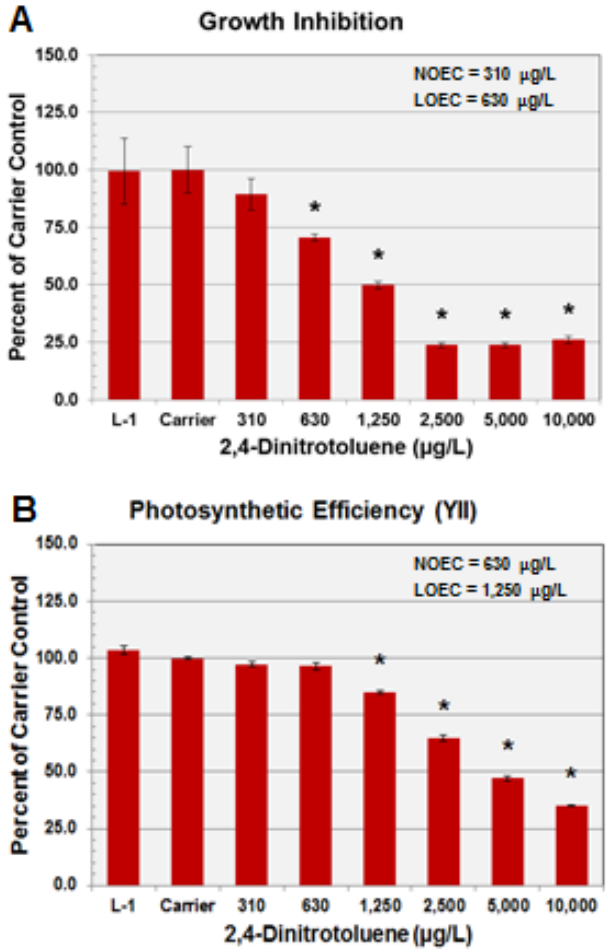


Figure 38. *Symbiodinium* Clade B cultures exposed to various concentrations of 2,4-DNT for 96 h. Bars show treatment means with ±1 SEM. Treatment means with * differed significantly at α = 0.05, one-way ANOVA followed by Dunnett's test for multiple comparisons versus the carrier control. A) Growth inhibition as determined by direct cell counts. B) Photosynthetic efficiency (Y(II)) measured using an Imaging-PAM fluorometer. NOEC = no-observed-effect concentration. LOEC = lowest-observed-effect concentration.

Summary of Toxicity Testing of 2,4-Dinitrotoluene

Summary reference toxicity values are presented in the following two tables. Table 11 provides summary information for NOEC and LOEC values that were statistically significant ($P<0.05$) different from control treatments. Table 12 provides summary information for effect concentration values for either lethality (LC) or sub-lethal effects (EC) for all testing of coral for 2,4-DNT.

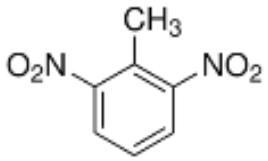
Table 11. Summary of no-observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) for all experiments exposing coral specimens to 2,4-DNT. ND=Not Determined.

Test Organism	NOEC 2,4 Dinitrotoluene	LOEC 2,4 Dinitrotoluene
Porites divaricata Calicoblast Cells – light 4 h	0.5 µg/L	5 µg/L
Porites divaricata Gastrodermal Cells – light 4 h	25 µg/L	100 µg/L
Pocillopora damicornis Calicoblast Cells – light 4 h	5 µg/L	25 µg/L
Pocillopora damicornis Gastrodermal Cells – light 4 h	0.1 µg/L	0.5 µg/L
Symbiodinium Clade B – 96 h *Pocillopora damicornis* Growth Inhibition	310 µg/L	630 µg/L
Symbiodinium Clade B – 96 h *Pocillopora damicornis* Photosynthetic Efficiency (YII)	630 µg/L	1,250 µg/L
Porites divaricata Fragment Total Porphyrin – 96 h	ND	ND

Table 12 Summary of lethal (LC_{50}, LC_{20}) and effect concentrations (EC_{50}, EC_{20}) for all experiments exposing coral specimens to 2,4-DNT. ND=Not Determined.

Test Organism	LC_{50} 2,4 Dinitrotoluene	LC_{20} 2,4 Dinitrotoluene
Porites divaricata Calicoblast Cells – light 4 h	5818 µg/L 95% CI = 3552-9771 µg/L	79 µg/L 95% CI = 32.4-157 µg/L
Porites divaricata Gastrodermal Cells – light 4 h	3123 µg/L 95% CI = 1865-5241 µg/L	52 µg/L 95% CI = 20-109 µg/L
Pocillopora damicornis Calicoblast Cells – light 4 h	1284 µg/L 95% CI = 797-2013 µg/L	15 µg/L 95% CI = 5.8-31.5 µg/L
Pocillopora damicornis Gastrodermal Cells – light 4 h	564.7 µg/L 95% CI = 298.5-1016 µg/L	2 µg/L 95% CI = 0.46-5.7 µg/L
	EC_{50}	EC_{20}
Symbiodinium Clade B from *Pocillopora damicornis* – 96 h Growth Inhibition	1315 µg/L 95% CI = 880-1964 µg/L	314 µg/L 95% CI = 157-630 µg/L
Symbiodinium Clade B- 96 h *Pocillopora damicornis* Photosynthetic Efficiency (YII)	4814.5 µg/L 95% CI = 4347-5331 µg/L	1381 µg/L 95% CI = 1138-1675 µg/L
Porites divaricata Fragment Total Porphyrin – 96 h	ND	ND

2,6-Dinitrotoluene



2,6-DNT

Background

2,6-Dinitrotoluene (CAS# 606-20-2; IUPAC 2-methyl-3,5-dinitroaniline, other synonyms: 2,6-DNT, 1,3-dinitro-2-methylbenzene; MW: 182.134 g/mol; color/form: yellow to red/crystals) is a major breakdown product of TNT as well as being used as an intermediate in the production of toluene diisocynate and polyurethanes in industry (e.g., waterproofing agent, plasticizer, and propellant). Its solublility is 123 mg/L at 19°C and 217 mg/L at 30°C in 33.1 ppt seawater (Prak and O'Sullivan 2007). This nitroaromatic compound can be introduced into the aquatic environment through disposal of raw materials from the manufacturing process, from the corrosion of unexploded ordinance and runoff from coastal artillery ranges (Prak and O'Sullivan 2007; O'Sullivan et al. 2011). It has been detected in seawater samples in Dokai Bay, Japan and a military munitions disposal area in Ordnance Reef Wai'nane, Hawaii. Nipper et al. (2009) indicated range EC_{50} values of 2.7-90 µmol/L for freshwater or marine algae and EC_{50} values for sea urchin embryo development and fertilization of 6.7 mg/L and 84 mg/L, respectively (Nipper et al. 2001).

Coral Cell Toxicity Testing

A six-point concentration gradient from 5 to 10,000 µg/L of 2,6-DNT was used for the exposures of calicoblast cells from *Porites lobata*, *Porites divaricata* and *Pocillopora damicornis*. An eight-point concentration gradient from 5 to 75,000 µg/L of 2,6-DNT was used for the exposures of gastrodermal cells from *P. lobata*, *P. divaricata* and *P. damicornis*. Primary cells were isolated and cultured as described in the methods and plated into 24-well Teflon® plates at approximately $6.5x10^5$ to $1.0x10^6$ cells per well. Cells were exposed for 4 h in the light (295 µmol m^{-2} s^{-1}) at 25°C. Cell viability counts were performed on each well using the trypan blue exclusion dye viability assay as the endpoint for effects characterization.

Observed-effect concentrations (NOEC and LOEC)

Exposure of ***Porites lobata*** **calicoblast** cells to various concentrations of 2,6-DNT showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison test found significant differences ($P<0.05$) among treatments versus the control group. The NOEC was 500 µg/L (500 ppb) and the LOEC was 2,500 µg/L (2.5 ppm) (Fig. 39A; Table 13).

Porites lobata **gastrodermal** cells showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's

multiple comparison test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was 25 µg/L (25 ppb) and the LOEC was 100 µg/L (100 ppb) (Fig. 39B; Table 13).

Exposure of ***Porites divaricata*** **calicoblast** cells to various concentrations of 2,6-DNT showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was 25 µg/L (25 ppb) and the LOEC was 100 µg/L (100 ppb) (Fig. 39C; Table 13).

Porites divaricata **gastrodermal** cells showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was 5 µg/L (5 ppb) and the LOEC was 25 µg/L (25 ppb) (Fig. 39D; Table 13).

Exposure of ***Pocillopora damicornis*** **calicoblast** cells to various concentrations of 2,6-DNT showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was 100 µg/L (100 ppb) and the LOEC was 500 µg/L (500 ppb) (Fig. 39E; Table 13).

Pocillopora damicornis **gastrodermal** cells showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was 5 µg/L (5 ppb) and the LOEC was 25 µg/L (25 ppb) (Fig. 39F; Table 13).

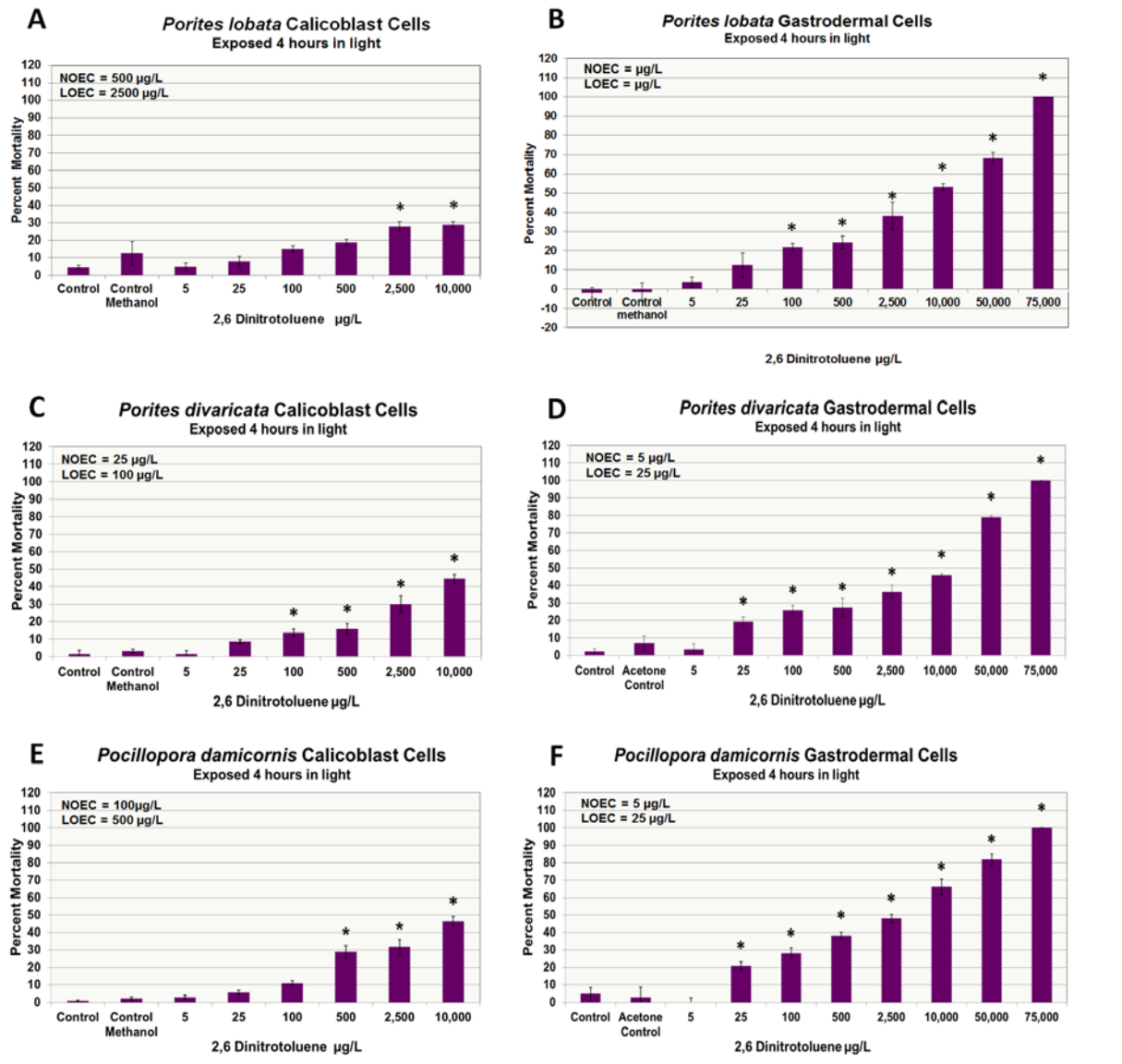


Figure 39. Percent mortality of *Porites lobata*, *Porites divaricata* and *Pocillopora damicornis* cells exposed to various concentrations of 2,6-DNT. n=4 replicates per treatment. Bars show treatment means with ±1 SEM. Treatment means with * differed significantly at $\alpha = 0.05$, one-way ANOVA followed by Dunnett's test for multiple comparisons versus the carrier control. A) *P. lobata* calicoblast cells. B) *P. lobata* gastrodermal. C) *P. divaricata* calicoblast cells. D) *P. divaricata* gastrodermal cells. E) *P. damicornis* calicoblast cells. F) *P. damicornis* gastrodermal cells. NOEC = no-observed-effect concentration. LOEC = lowest-observed effect concentration.

LC_{50} and LC_{20}

Porities lobata, Porities divaricata and *Pocillopora damicornis* calicoblast and gastrodermal cell mortality data for 2,6-DNT exposures were subjected to PROBIT analysis to determine the median lethal concentration (LC_{50}) and 20% lethal concentration (LC_{20}) values and confidence intervals.

The LC_{50} for ***Porites lobata*** **calicoblast cells** was 105,124 µg/L (105 ppm) and the LC_{20} was 948 µg/L (948 ppb) (Fig. 40A; Table 14). The LC_{50} for ***Porites lobata*** **gastrodermal** cells was 4,748 µg/L (4.74 ppm) and the LC_{20} was 216 µg/L (216 ppb) (Fig. 40B; Table 14).

The LC_{50} for ***Porites divaricata*** **calicoblast cells** was 16,557 µg/L (16.5 ppm) and the LC_{20} was 631 µg/L (631 ppb) (Fig. 37C; Table 12). The LC_{50} for ***Porites divaricata*** **gastrodermal** cells was 3,699 µg/L (3.7 ppm) and the LC_{20} was 137 µg/L (137 ppb) (Fig. 40D; Table 14).

The LC_{50} for ***Pocillopora damicornis*** **calicoblast** cells was 11,075 µg/L (11.5 ppm) and the LC_{20} was 463 µg/L (463 ppb) (Fig. 40E; Table 14). The LC_{50} of ***Pocillopora damicornis*** **gastrodermal** cells was 1,844 µg/L (1.8 ppm) and the LC_{20} was 71 µg/L (71 ppb) (Fig. 40F; Table 14).

Each species tested displayed a different response to the exposure conditions. The relative sensitivities to 2,6-DNT were *P. damicornis* > *P. divaricata* > *P. lobata,* with *P. lobata* being the most tolerant of this munitions breakdown product. These findings are the first to use an *in vitro* coral cell-based toxicity test for dose-response characterization and comparisons among three shallow-water coral species to 2,6-DNT. Finding differing responses among the three species is important because this begins to lay a foundation for predicting possible ecological impacts and risk that may differ across sites depending on the species present at any particular reef site.

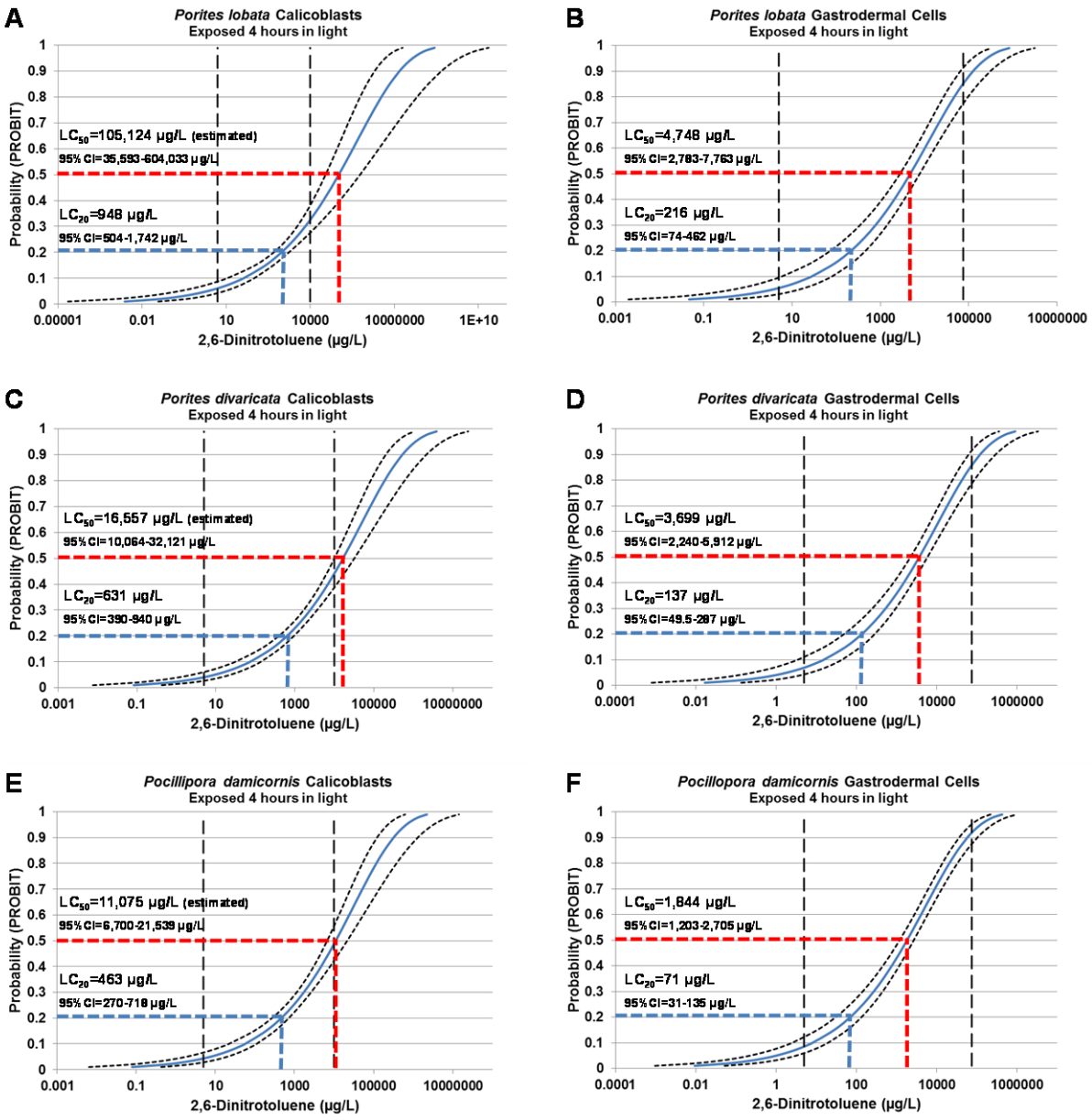


Figure 40. Results of PROBIT analyses for determining lethal concentration (LC_{50} and LC_{20}) values for coral cells exposed to 2,6-DNT in light conditions. A) *P. lobata* calicoblast cells. B) *P. lobata* gastrodermal cells. C) *P. divaricata* calicoblast cells. D) *P. divaricata* gastrodermal cells. E) *P. damicornis* calicoblast cells. F) *P. damicornis* gastrodermal cells. Vertical bars indicate dose range tested.

Symbiodinium sp. Clade B Culture Toxicity Testing

Symbiodinium Clade B was exposed to a geometric dose range (ratio=2) of 2,6 dinitrotoluene from 1,560 to 50,000 µg/L. Cell growth was monitored as one endpoint. No significant difference was found between the carrier control and media control. Thus the data were evaluated as percent of carrier control at 96 h. Significant effects were determined by a one-way ANOVA with the post-hoc Dunnett's multiple comparison test for treatments versus carrier control. The NOEC was 12,500 µg/L and the LOEC was 25,000 µg/L (Fig. 41A; Table 13). A modified PROBIT analysis using a non-linear regression estimated the EC_{50} as 22,970 µg/L.

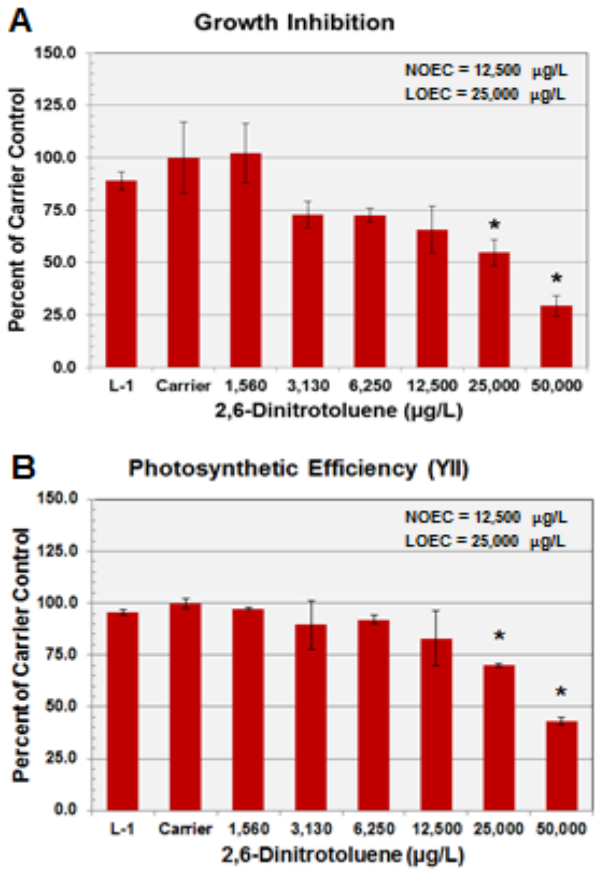


Figure 41. *Symbiodinium* Clade B cultures exposed to various concentrations of 2,6-DNT after 96 h. Bars show treatment means with ±1 SEM. Treatment means with * differed significantly at α = 0.05, one-way ANOVA followed by Dunnett's test for multiple comparisons versus the carrier control. A) Growth inhibition as determined by direct cell counts. B) Photosynthetic efficiency (Y(II)) measured using an Imaging-PAM fluorometer. NOEC=no-observed-effect concentration. LOEC=lowest-observed-effect concentration.

Photosynthetic efficiency of the *Symbiodinium* was used as a measure of the health condition of the photosystems. Significant effects were determined by a one-way ANOVA with the post-hoc Dunnett's multiple comparison test for treatments versus carrier control. The NOEC was 12,500 µg/L and the LOEC was 25,000 µg/L (Fig. 41B; Table 13). Effective quantum yield (Y(II)) was used to calculate EC_{50} values which were estimated as 27,040 µg/L using a non-linear regression. This parameter mirrored the cell count data. LC Mass spectroscopy by EPA method 3535A indicated that the compound remained stable for the duration of the experiment.

Summary of Toxicity Testing of 2,6-Dinitrotoluene

Summary reference toxicity values are presented in the following two tables. Table 13 provides summary information for NOEC and LOEC values that were statistically significant ($P<0.05$) different from control treatments. Table 14 provides summary information for effect concentration values for either lethality (LC) or sub-lethal effects (EC) for all testing of coral for 2,6-DNT.

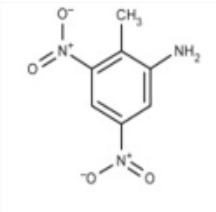
Table 13. Summary of no-observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) for all experiments exposing coral specimens to 2,6-DNT.

Test Organism	NOEC 2,6 Dinitrotoluene	LOEC 2,6 Dinitrotoluene
Porites lobata Calicoblast Cells – light 4 h	500 µg/L	2,500 µg/L
Porites lobata Gastrodermal Cells – light 4 h	25µg/L	100 µg/L
Porites divaricata Calicoblast Cells – light 4 h	25 µg/L	100 µg/L
Porites divaricata Gastrodermal Cells – light 4 h	5 µg/L	25 µg/L
Pocillopora damicornis Calicoblast Cells – light 4 h	100 µg/L	500 µg/L
Pocillopora damicornis Gastrodermal Cells – light 4 h	5 µg/L	25 µg/L
Symbiodinium Clade B – 96 h *Pocillopora damicornis* Growth Inhibition	12,500 µg/L	25,000 µg/L
Symbiodinium Clade B – 96 h *Pocillopora damicornis* Photosynthetic Efficiency (YII)	12,500 µg/L	25,000 µg/L

Table 14. Summary of lethal concentrations (LC_{50}, LC_{20}) and effect concentrations (LC_{50}, LC_{20}) for all experiments exposing coral specimens to 2,6-DNT.

Test Organism	LC_{50} 2,6 Dinitrotoluene	LC_{20} 2,6 Dinitrotoluene
Porites lobata Calicoblast Cells – light 4 h	105,124 µg/L (estimated) 95% CI = 35,593-604,033 µg/L	948 µg/L 95% CI = 504-1,742µg/L
Porites lobata Gastrodermal Cells– light 4 h	4,748 µg/L 95% CI = 2,783-7,763 µg/L	216 µg/L 95% CI = 74-462 µg/L
Porites divaricata Calicoblast Cells – light 4 h	16,557 µg/L (estimated) 95% CI = 10,064-32,121 µg/L	631 µg/L 95% CI = 390-940 µg/L
Porites divaricata Gastrodermal Cells – light 4 h	3,699 µg/L 95% CI = 2,240-5,912 µg/L	137 µg/L 95% CI = 49.5-287 µg/L
Pocillopora damicornis Calicoblast Cells – light 4 h	11,075 µg/L (estimated) 95% CI = 6,700-21,539 µg/L	463 µg/L 95% CI = 270-718 µg/L
Pocillopora damicornis Gastrodermal Cells – light 4 h	1,844 µg/L 95% CI = 1,203-2,705 µg/L	70.7 µg/L 95% CI = 31-135 µg/L
	EC_{50}	EC_{20}
Symbiodinium Clade B – 96 h *(Pocillopora damicornis)* Growth Inhibition	22,292 µg/L 95% CI =10,750-46,226 µg/L	4,135 µg/L 95% CI = 1,064-16,066 µg/L
Symbiodinium Clade B - 96 h *(Pocillopora damicornis)* Photosynthetic Efficiency (YII)	45,516 µg/L 95% CI = 33,119-62,554 µg/L	17,749 µg/L 95% CI = 10,779-29,224 µg/L

2-Amino-4,6-Dinitrotoluene



2-ADNT

Background

2-Amino-4,6-Dinitrotoluene (CAS# 35572-78-2; IUPAC, 2-methyl-3,5-dinitroaniline; other synonyms: 2-ADNT, 3,5-dinitro-o-toluidine, 4,6-dinitrotoluene-2-amine; MW: 197.15 g/mol; color/form: ND/crystals) is a reduction product of TNT as well as a major microbial breakdown product of TNT. It is soluble in water at 38 mg/L at 20°C. Its solubility in seawater is unknown. U.S. Army reports (2005) indicated 1,394-2,240 mg/kg range LD_{50} for rats and 1,522-1,722 mg/kg for mice. Nipper et al. (2009) indicates a 96 h survival LC_{50} of 75.1-76.6 µmol/L, in contrast marine arthropods (*Tigriopus californicus*) and molluscs (*Crassostrea gigas*) demonstrate LC_{50} values > 253 µmol/L. Values for marine algae are not reported, however freshwater algae (*Pseudokirchneriella subcapitata*) demonstrate an IC_{50} of 12.9 µmol/L.

Coral Cell Toxicity Testing

Coral cell toxicity testing was not conducted for 2-amino-4,6-dinitrotoluene.

Symbiodinium sp. Clade B Culture Toxicity Testing

Symbiodinium Clade B was exposed to a geometric dose range (ratio=2) of 2-amino-4,6-dinitrotoluene (2-ADNT) from 630 to 20,000 µg/L. Cell growth was monitored as one endpoint. No significant difference was found between the carrier control and media control. Thus the data were evaluated as percent of carrier control at 96 h. Significant effects ($p<0.05$) were determined by a one-way ANOVA with the post-hoc Dunnett's multiple comparisons test for treatments versus carrier control. The NOEC was 630 µg/L and the LOEC was 1,250 µg/L (Fig. 42A). A modified PROBIT using a non-linear regression analysis estimated the EC_{50} as 4,059 µg/L.

Photosynthetic efficiency of the *Symbiodinium* was used as a measure of the health condition of the photosystems. Significant effects ($p<0.05$) were determined by a one-way ANOVA with the post-hoc Dunnett's multiple comparison for treatments versus carrier control. The NOEC was <630 µg/L and the LOEC was 630 µg/L (Fig. 42B). Effective quantum yield (Y(II)) was used to calculate EC_{50} values which were estimated as 10,206 µg/L using a non-linear regression.

Photosynthetic efficiency and growth inhibition of the *Symbiodinium* cells were affected similarly by 2-ADNT exposure. However, in comparison photosynthetic efficiency appeared slightly more sensitive to 2,4-DNT and 2,3-DNT than TNT, denoting a differing order of relative toxicity.

The fact that toxicity values for cell growth and photosynthetic efficiency appeared to correlate was an important observation because this provides an important piece of data supporting the use of photosynthetic efficiency as a proxy for relative health of corals in the field. A regression analysis was conducted for 2-ADNT (Fig. 43) as well as TNT. The analysis indicated a high correlation for 2-ADNT (Adjusted R-square=0.84), meaning that 84% of the variation is explained by this association. Continuing to evaluate the correlations between photosynthetic efficiency and other health parameters in coral fragments undergoing toxicity testing may give information as to whether non-invasive PAM fluorometry is a valid biomarker for military base environmental managers to use in monitoring corals at their sites and the efficacy of this method as part of this project's transition plan.

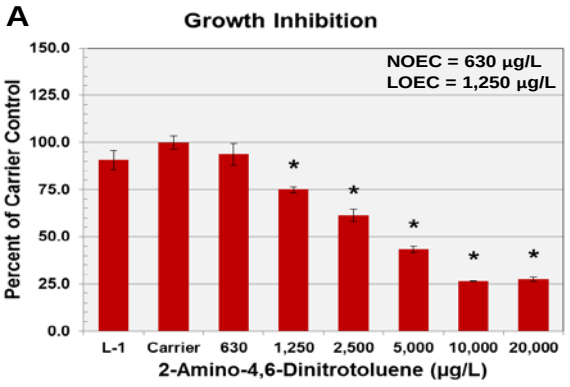


Figure 42. *Symbiodinium* Clade B cultures exposed to various concentrations of 2-ADNT for 96 h. Bars show treatment means with ±1 SEM. Treatment means with * differed significantly at $\alpha = 0.05$, one-way ANOVA followed by Dunnett's test for multiple comparisons versus the carrier control. A) Growth inhibition as determined by direct cell counts. B) Photosynthetic efficiency (Y(II)) measured using an Imaging-PAM fluorometer. NOEC = no-observed-effect concentration. LOEC=lowest-observed-effect concentration.

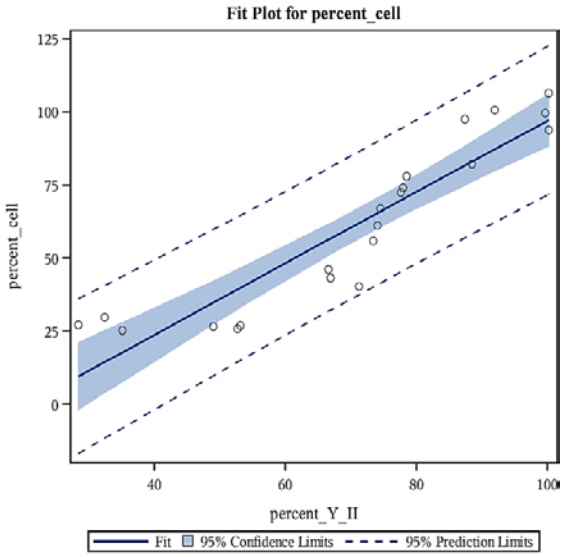


Figure 43. Regression analysis of *Symbiodium* growth inhibition in relation to photosynthetic efficiency (Y(II)) exposed to 2-ADNT after 96 h. Cell growth inhibition demonstrates a strong correlation to Y(II) (Adjusted R-square=0.84).

Summary of Toxicity Testing of 2-Amino-4,6-Dinitrotoluene

Summary reference toxicity values are presented in the following two tables. Table 15 provides summary information for NOEC and LOEC values that were statistically significant ($P<0.05$) different from control treatments. Table 16 provides summary information for effect concentration values for either lethality (LC) or sub-lethal effects (EC) for all testing of coral for 2-ADNT.

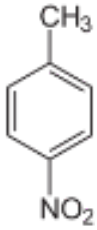
Table 15. Summary of no-observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) for all experiments exposing coral specimens to 2-ADNT. ND=Not Determined.

Test Organism	NOEC 2-Amino-4,6-Dinitrotoluene	LOEC 2-Amino-4,6-Dinitrotoluene
Porites divaricata Calicoblast Cells	ND	ND
Porites divaricata Gastrodermal Cells	ND	ND
Pocillopora damicornis Calicoblast Cells	ND	ND
Pocillopora damicornis Gastrodermal Cells	ND	ND
Symbiodinium Clade B – 96 h *Pocillopora damicornis* Growth Inhibition	630 µg/L	1,250 µg/L
Symbiodinium Clade B – 96 h (*Pocillopora damicornis*) Photosynthetic Efficiency (YII)	<630 µg/L	630 µg/L
Pocillopora damicornis Fragment Total Porphyrin – 96 h	ND	ND

Table 16. Summary of lethal concentrations (LC_{50}, LC_{20}) and effect concentrations (EC_{50}, EC_{20}) for all experiments exposing coral specimens to 2-ADNT. ND=Not Determined.

Test Organism	LC_{50} 2-Amino-4,6-Dinitrotoluene	LC_{20} 2-Amino-4,6-Dinitrotoluene
Porites divaricata Calicoblast Cells	ND	ND
Porites divaricata Gastrodermal Cells	ND	ND
Pocillopora damicornis Calicoblast Cells	ND	ND
Pocillopora damicornis Gastrodermal Cells	ND	ND
	EC_{50}	EC_{20}
Symbiodinium Clade B – 96 h (*Pocillopora damicornis*) Growth Inhibition	4,059 µg/L 95% CI = 3,146-5,238 µg/L	915 µg/L 95% CI = 584-1,434 µg/L
Symbiodinium Clade B- 96 h (*Pocillopora damicornis*) Photosynthetic Efficiency (YII)	10,206 µg/L 95% CI = 8,427-12,362 µg/L	1,802 µg/L 95% CI = 1,262-2,572 µg/L
Pocillopora damicornis Fragment Total Porphyrin – 96 h	ND	ND

4-Nitrotoluene



4-NT

Background

4-Nitrotoluene (CAS# 99-99-0; IUPAC, 1-methyl-4-nitrobenzene; other synonyms: 4-NT, 4-methylnitrobenaene, 4-nitrotoluol, p-Nitrophenylmethane; MW: 137.14 g/mol; color/form: yellow/rhombic crystals) is used in the production of dyestuff, pesticides, rubber, and explosives. Its solubility is 230 mg/L at 19°C and 339 mg/L at 30°C in 33.1 ppt seawater (Prak and O'Sullivan 2007). EC_{50} values for freshwater or marine algae are reported to range between 24.1-182.0 µmol/L (Zhao et al. 1997; Nipper et al. 2009) while the freshwater arthropod, *Daphnia magna*, has EC_{50} values ranging from 51.3 µmol/L for reproduction to 137.8 µmol/L for immobility or survival endpoints (Nipper et al. 2009).

Coral Cell Toxicity Testing

Coral cell toxicity testing was not conducted for 4-nitrotoluene.

Symbiodinium sp. Clade B Culture Toxicity Testing

Symbiodinium Clade B was exposed to a geometric dose range (ratio=2) of 4-nitrotoluene (4-NT) from 1,560 to 50,000 µg/L. Cell growth was monitored as one endpoint. No significant difference was found between the carrier control and media control. Thus the data were evaluated as percent of carrier control at 96 h. Significant effects ($p<0.05$) were determined by a one-way ANOVA with the post-hoc Dunnett's multiple comparisons test for treatments versus carrier control. The NOEC was 25,000 µg/L and the LOEC was 50,000 µg/L (Fig. 44A; Table 17). A modified PROBIT using a non-linear regression analysis estimated the EC_{50} as >45,000 µg/L (Table 18).

Photosynthetic efficiency of the *Symbiodinium* was used as a measure of the health condition of the photosystems. Significant effects ($p<0.05$) were determined by a one-way ANOVA with the post-hoc Dunnett's multiple comparisons test for treatments versus carrier control. The NOEC was 25,000 µg/L and the LOEC was 50,000 µg/L (Fig. 44B; Table 17). Effective quantum yield (Y(II)) was used to calculate estimated EC_{50} values which were estimated as >89,000 µg/L using a non-linear regression (Table 18). This parameter mirrored the cell count data.

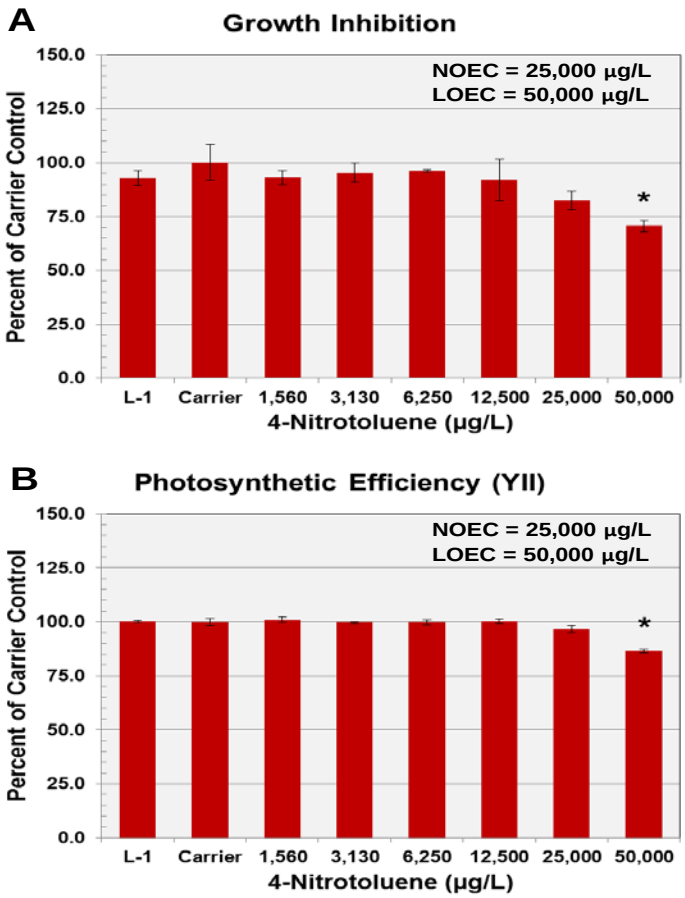


Figure 44. *Symbiodinium* Clade B cultures exposed to various concentrations of 4-NT for 96 h. Bars show treatment means with ±1 SEM. Treatment means with * differed significantly at $\alpha = 0.05$, one-way ANOVA followed by Dunnett's test for multiple comparisons versus the carrier control. A) Growth inhibition as determined by direct cell counts. B) Photosynthetic efficiency (Y(II)) measured using an Imaging-PAM fluorometer. NOEC=no-observed-effect concentration. LOEC=lowest-observed-effect concentration.

Summary of Toxicity Testing of 4-Nitrotoluene

Summary reference toxicity values are presented in the following two tables. Table 17 provides summary information for NOEC and LOEC values that were statistically significant ($P<0.05$) different from control treatments. Table 18 provides summary information for effect concentration values for either lethality (LC) or sub-lethal effects (EC) for all testing of coral for 4-NT.

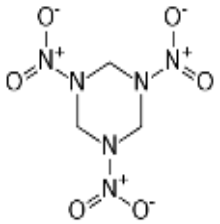
Table 17. Summary of no-observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) for all experiments exposing coral specimens to 4-NT. ND=Not Determined.

Test Organism	NOEC 4-Nitrotoluene	LOEC 4-Nitrotoluene
Porites divaricata Calicoblast Cells	ND	ND
Porites divaricata Gastrodermal Cells	ND	ND
Pocillopora damicornis Calicoblast Cells	ND	ND
Pocillopora damicornis Gastrodermal Cells	ND	ND
Symbiodinium Clade B – 96 h *Pocillopora damicornis* Growth Inhibition	25,000 µg/L	50,000 µg/L
Symbiodinium Clade B – 96 h *Pocillopora damicornis* Photosynthetic Efficiency (YII)	25,000µg/L	50,000 µg/L
Pocillopora damicornis Fragment Total Porphyrin – 96 h	ND	ND

Table 18. Summary of lethal concentrations (LC_{50}, LC_{20}) and effect concentrations (EC_{50}, EC_{20}) for all experiments exposing coral specimens to 4-NT. ND=Not Determined.

Test Organism	LC_{50} 4-Nitrotoluene	LC_{20} 4-Nitrotoluene
Porites divaricata Calicoblast Cells	ND	ND
Porites divaricata Gastrodermal Cells	ND	ND
Pocillopora damicornis Calicoblast Cells	ND	ND
Pocillopora damicornis Gastrodermal Cells	ND	ND
	EC_{50}	EC_{20}
Symbiodinium Clade B – 96 hr *Pocillopora damicornis* Growth Inhibition	116,083 µg/L 95% CI = 44,891-300,173 µg/L	35,715 µg/L 95% CI = 22,430-56,869 µg/L
Symbiodinium Clade B - 96 hr *Pocillopora damicornis* Photosynthetic Efficiency (YII)	137,900 µg/L 95% CI = 88,940-213,818 µg/L	62,814 µg/L 95% CI = 54,555-72,324 µg/L
Pocillopora damicornis Fragment Total Porphyrin – 96 h	ND	ND

1,3,5-Trinitroperhydro-1,3,5-triazine



RDX

Background

RDX, (CAS# 121-82-4; IUPAC, 1,3,5-Trinitroperhydro-1,3,5-triazine, other names Cyclonite, Royal Demolition Explosive, Research Department Composition X; MW=222.12 g/mol; color/form: white, crystalline). Nipper et al. (2001) found that the explosive enters the water by use, storage, improper disposal and incineration. Solubility is reported as 43.2 mg/L in water at 25°C (Waisner et al. 2002). Solubility in seawater is reported as 13.93 mg/L at 20°C, 19.77 mg/L at 25°C and 27.95 mg/L at 30°C (Kholod 2011). Nipper et al. (2009) indicated EC_{50} values >144 and IC_{50} values >181.0 µmol/L and the U.S. Geological Survey (NFESC 2000) reported 8.8-11.0 µg/ml range values for freshwater or marine. Lotufo et al. (2010) determined the LC_{50} for the sheepshead minnow, *Cyprinodon variegatus*, to be 10.3 mg/L after 96 h. Nipper et al. (2001) reported the toxicity values for multiple marine organisms including marine alga (*Ulva fasciata*) zoospore germination; polychaete (*Dinophilus gyrociliatus*) survival; sea urchin (*Arbacia punctulata*) embryo development; redfish (*Sciaenops ocellatus*) embryo-larval survival. They determined EC_{50} values as 12 mg/L for *Ulva*; >49 mg/L for polychaete; sea urchin >75 mg/L; and >68 mg/L for redfish embryos and larvae. Nipper at al. (2001) also determined the RDX NOEC/LOEC values for *Ulva* as 9.2/15.7 mg/L, polychaete as 49/ >49 mg/L; sea urchin as 75/ >75 mg/L; and redfish as 68/ >68 mg/L. One cnidarian, *Hydra littoralis*, a freshwater anemone, had a reported 48 h LC_{50} value of 32 mg/L (Nipper et al. 2009).

Coral Cell Toxicity Testing

A six-point concentration gradient from 1 µg/L to 10,000 µg/L of RDX was used for the exposures of calicoblast and gastrodermal cells from *Porites divaricata* and *Pocillopora damicornis*. Primary cell cultures were isolated and cultured as described in the methods and plated into 24-well Teflon® plates at approximately $6.5x10^5$ to $1x10^6$ cells per well. Cells were exposed for 4 h in the light (295 µmol m^{-2} s^{-1}) at 25°C. Cell viability counts were performed on each well using the trypan blue exclusion dye viability assay as the endpoint for effects characterization.

Observed-effect concentrations (NOEC and LOEC)

Exposure of ***Porites divaricata*** **calicoblast** cells to various concentrations of RDX showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparisons test found significant

differences ($p<0.05$) among treatments versus the control group. The NOEC was 100 µg/L (100 ppb) and the LOEC was 500 µg/L (500 ppb) (Fig. 45A; Table 19).

Porites divaricata **gastrodermal** cells showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparisons test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC 500 µg/L (500 ppb) and the LOEC was 1,000 µg/L (1 ppm) (Fig. 45B; Table 19).

Exposure of ***Pocillopora damicornis*** **calicoblast** cells to various concentrations of RDX showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparisons test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was 100 µg/L (100 ppb) and the LOEC was 500 µg/L (500 ppb) (Fig. 45C; Table 19).

Pocillopora damicornis **gastrodermal** cells showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparisons test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was 1,000 µg/L (1 ppm) and the LOEC was 10,000 µg/L (10 ppm) (Fig. 45D; Table 19).

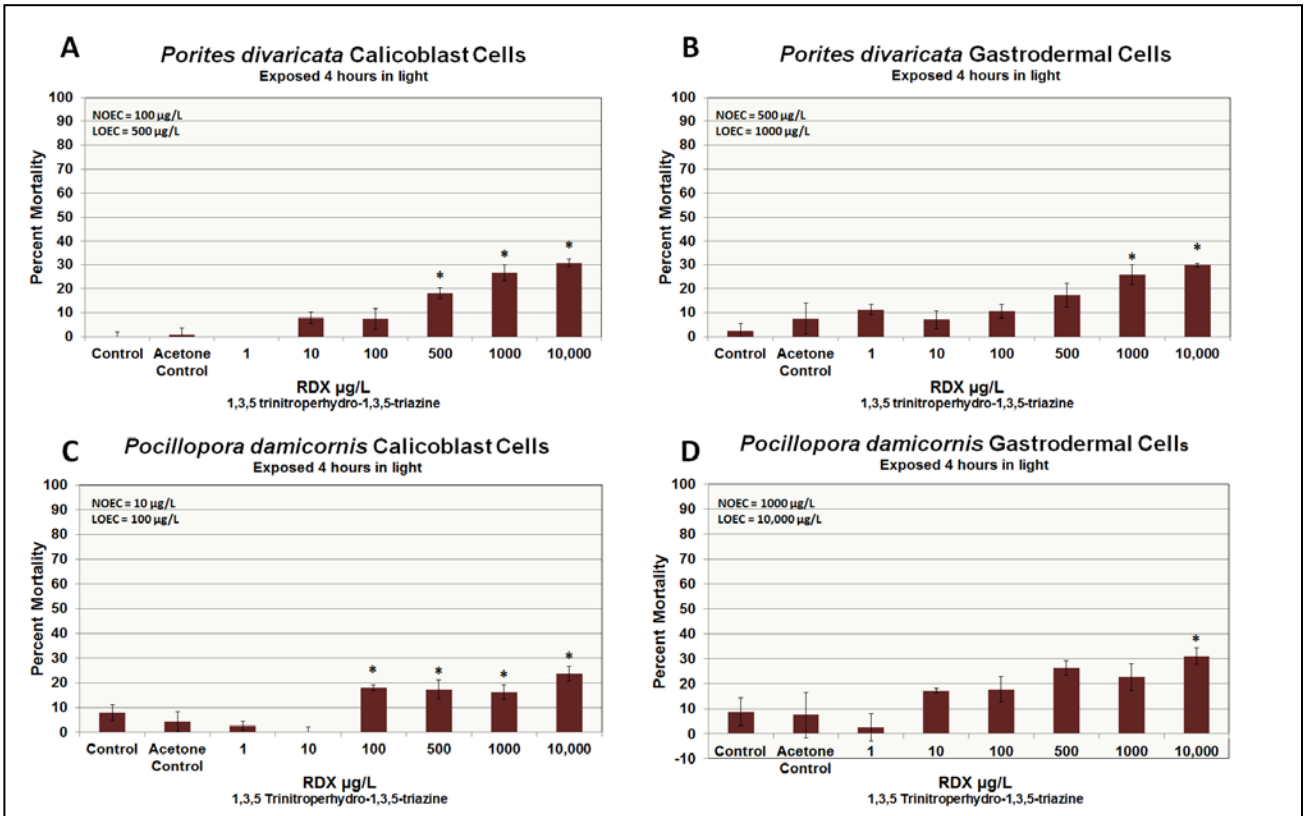


Figure 45. Percent mortality of *Porites divaricata* and *Pocillopora damicornis* cells exposed to various concentrations of RDX. n=4 replicates per treatment. Bars show treatment means with ±1 SEM. Treatment means with * differed significantly at $\alpha = 0.05$, one-way ANOVA followed by Dunnett's test for multiple comparisons test versus the carrier control. A) *P. divaricata* calicoblast cells. B) *P. divaricata* gastrodermal cells. C) *P. damicornis* calicoblast cells. D) *P. damicornis* gastrodermal cells. NOEC=no-observed-effect concentration. LOEC=lowest-observed effect concentration.

$\underline{LC_{50}}$ and $\underline{LC_{20}}$

The data did not reach at least 50% lethality required for PROBIT analysis. Therefore, since the data failed to generate valid models neither LC_{50} or LC_{20} values could not be determined for either coral species or cell type tested.

Symbiodinium sp. Clade B Culture Toxicity Testing

Symbiodinium Clade B was exposed to a geometric dose range (ratio=2) of RDX from 470 to 15,000 µg/L. Cell growth was monitored as one endpoint. No significant difference was found between the carrier control and media control. Thus the data were evaluated as percent of carrier control at 96 h. A one-way ANOVA with the post-hoc Dunnett's multiple comparisons test for treatments versus carrier control showed no significant difference between treatment groups and carrier controls. The NOEC was >15,000 µg/L and the LOEC was >15,000 µg/L (Fig. 46A). The data did not reach at least 50% effect therefore valid PROBIT models could not generated for determination of EC values.

Photosynthetic efficiency of the *Symbiodinium* was used as a measure of the health condition of the photosystems. No significant effect was found by a one-way ANOVA with the post-hoc Dunnett's multiple comparisons test for treatments versus carrier control. The NOEC was >15,000 µg/L and the LOEC was >15,000 µg/L (Fig. 46B). EC_{50} values could not be determined from the data as there were no significant differences among treatments based on effective quantum yield (Y(II)) This parameter mirrored the cell count data.

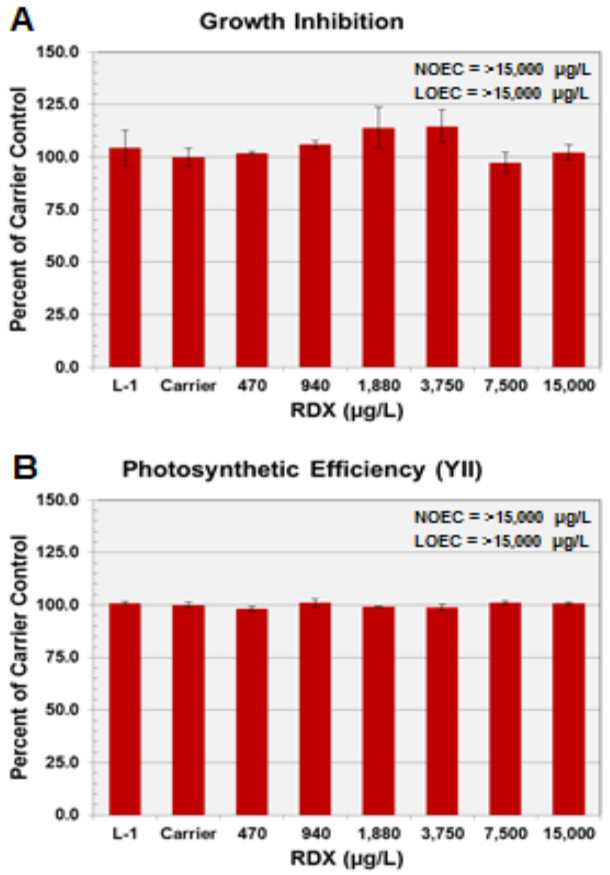


Figure 46. *Symbiodinium* Clade B cultures exposed to various concentrations of RDX for 96 h. Bars show treatment means with ±1 SEM. Treatment means with * differed significantly at α = 0.05, one-way ANOVA followed by Dunnett's test for multiple comparisons versus the carrier control. A) Growth inhibition as determined by direct cell counts. B) Photosynthetic efficiency (Y(II)) measured using an Imaging-PAM fluorometer. NOEC=no-observed-effect concentration. LOEC=lowest-observed-effect concentration.

Coral Fragment Exposures

Pocillopora damicornis fragments were exposed to RDX in a time-course design for 96 h. The experimental design involved seven replicated treatments with treatment concentrations of 250, 500, 1,000, 2,000, 4,000, 8,000 or 16,000 µg/L, plus a seawater and carrier (acetone) control with four replicates for each treatment and four fragments in each replicate for time point sampling. The dosages were based results from zooxanthellae testing as well as solubility limits in seawater.

Physio-score

No visual differences occurred during the 96 h exposure of *P. damicornis* to RDX at any concentration tested.

Histopathology

Fragments exposed to various concentrations of RDX for 96 h were examined for histopathological changes in tissues. Time zero fragments were sampled at the outset of setting up the experiment for comparison. Tissues from all treatments presented with normal histological features. The epidermis exhibited cuboidal to short columnar epithelial cells, zooxanthellae were intact and normally situated in the gastrodermis. However, for the highest concentration (16,000 ug/L) at 96 h there were noticeably more vacuolated cells present in the epidermis. The calicodermis appeared active with extensions and thin secretions of organic matrix used for attachment to skeleton. Mesoglea was thick and muscle fibers were well-formed. There were abundant nematocysts and spirocysts in the tentacles. Nematocysts and acidophilic granular gland cells were also present in the cnidoglandular bands of mesenterial filaments. Carrier controls showed minor changes with the epidermis becoming more columnar, with scattered mucocytes. Other structures were similar to the T0 samples. Gastrodermal cells appeared normal with well-formed normal nuclei (Fig. 47).

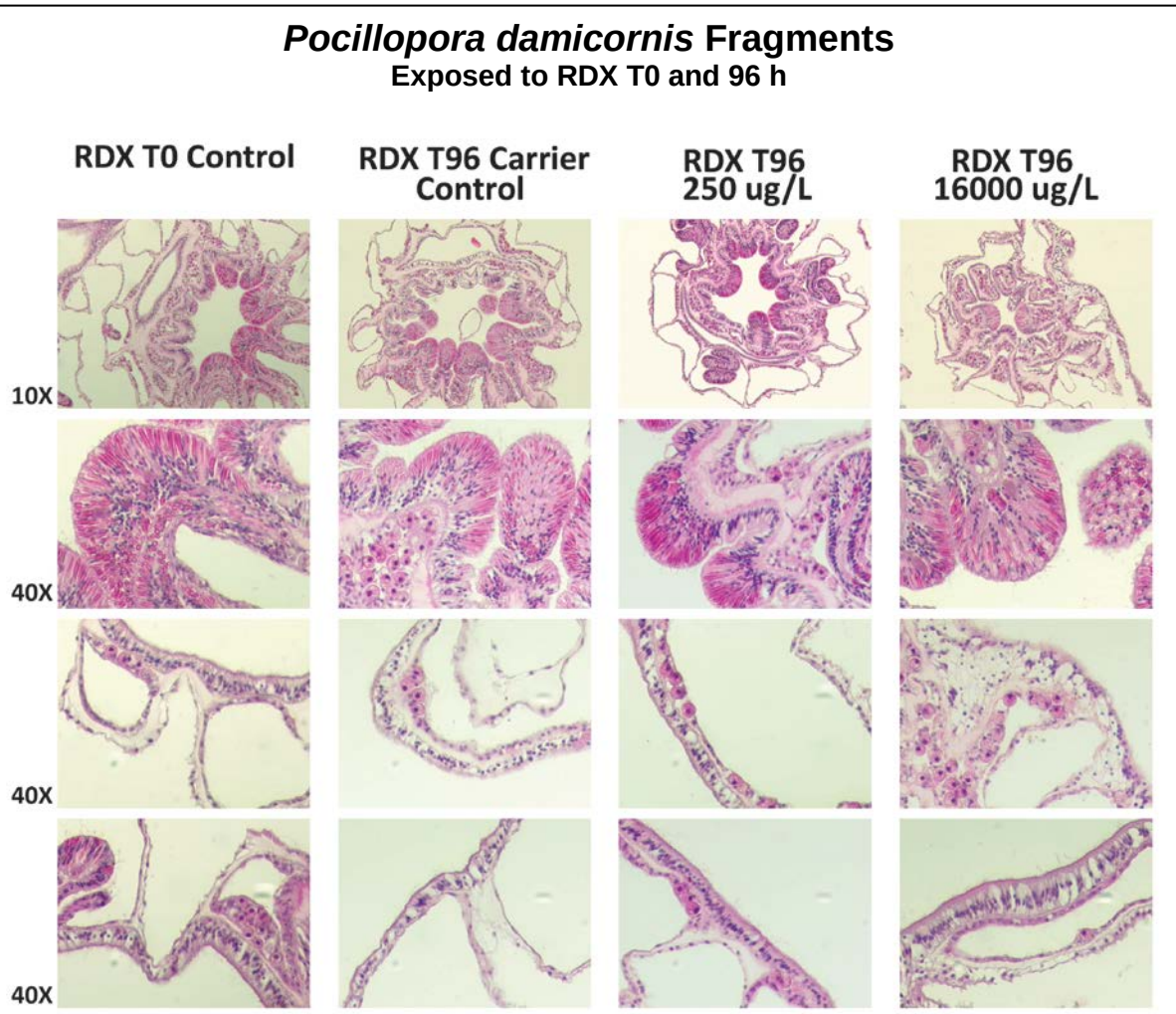


Figure 47. Histopathology of *Pocillopora damicornis* exposed to various concentrations of RDX for 96 h. Photomicrographs of hematoxylin and eosin stained tissues show a survey of the coral's epidermis, gastrodermis and calicodermis across treatments. The highest concentration (16,000 ug/L) at 96 h revealed noticeably more vacuolated cells in the epidermis than fragments from 0 h control, carrier control at 96 h, and lowest concentration (250 ug/L) at 96 h shown here.

PAM Fluorometric Analysis

Pocillopora damicornis fragments exposed to various concentrations of RDX for 96 h were analyzed by PAM fluorometry at each time point. A single fragment from each replicate was designated prior to dosing, and this same fragment was measured continuously throughout the experiment. At the time of analysis, the fragments were dark adapted for 5 min and then exposed to a saturation pulse to determine the maximum photosynthetic quantum yield (Fv/Fm) (Fig. 48). Other common PAM parameters such as Y(II), Y(NO), Y(NPQ) were not determined due to time constraints and possible stress imposed on the coral fragments from prolonged measurements.

On average Fv/Fm decreased over time for all treatments including the controls (Fig. 49). However, there were no significant differences in Fv/Fm values observed between treatments at any time point. The inconsistently high value measured in the carrier control at 14 h (Fig. 49), was most likely due to an instrumental error and is not considered a legitimate effect of the dosing regimen.

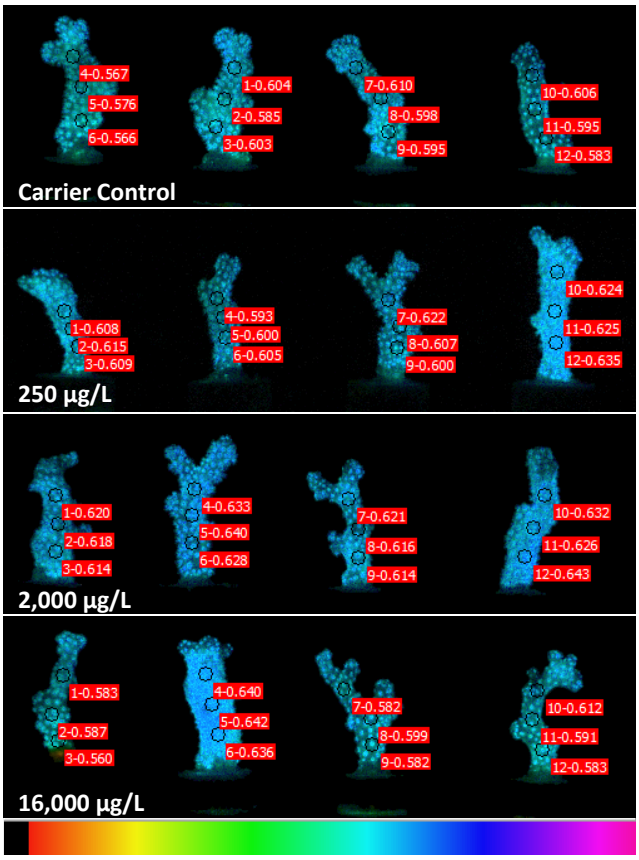


Figure 48. Maximum photosynthetic quantum yield (Fv/Fm) of *Pocillopora damicornis* fragments exposed to RDX for 96 h. Measurements were taken with a Waltz MAXI-Imaging PAM fluorometer after 5 min of dark-adaption. Three areas of interest were positioned on each replicate to average values over the whole fragment.

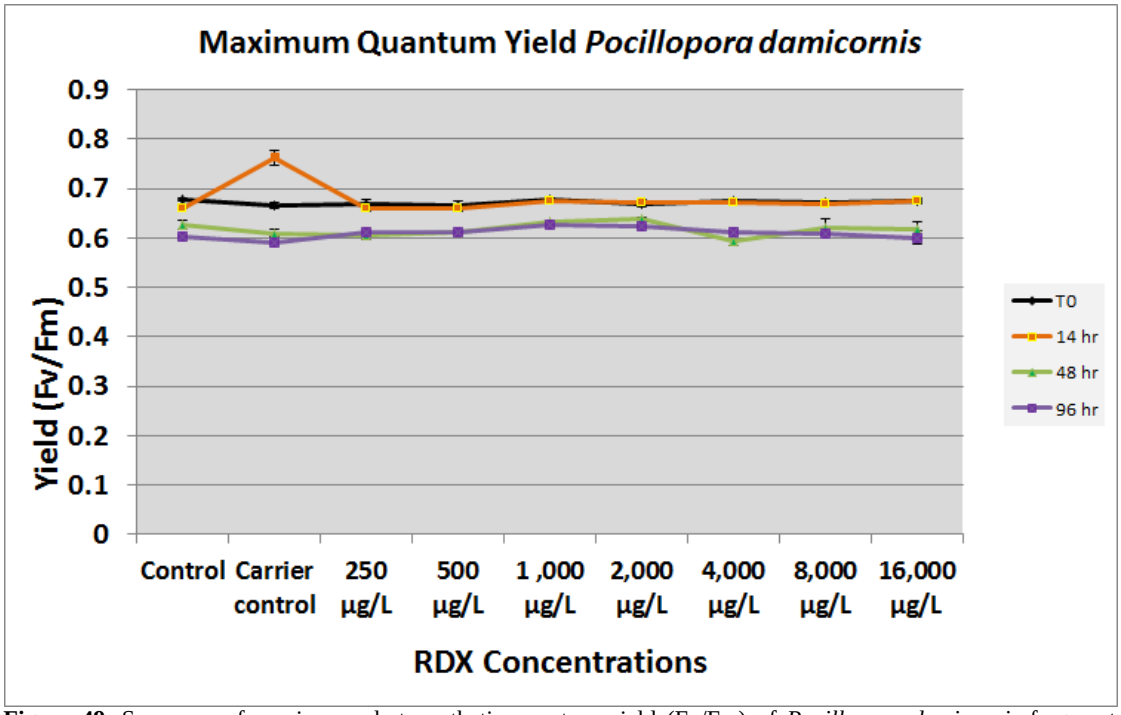


Figure 49. Summary of maximum photosynthetic quantum yield (Fv/Fm) of *Pocillopora damicornis* fragments exposed to RDX across all time points. Measurements were taken with a Waltz MAXI-Imaging PAM fluorometer after 5 min of dark-adaption. Measures were derived from three areas of interest on each replicate to average values over the whole fragment.

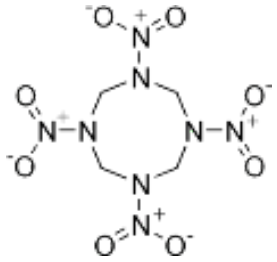
Summary of Toxicity Testing of RDX

Summary reference toxicity values are presented in the following two tables. Table 19 provides summary information for NOEC and LOEC values that were statistically significant ($P<0.05$) different from control treatments. No effect concentration values for either lethality (LC) or sub-lethal effects (EC) could be determined for RDX at the concentrations tested.

Table 19. Summary of no-observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) for all experiments exposing coral specimens to RDX. ND=Not Determined.

Test Organism	NOEC RDX	LOEC RDX
Porites divaricata Calicoblast Cells – light 4 hr	100 µg/L	500 µg/L
Porites divaricata Gastrodermal Cells – light 4 hr	500 µg/L	1,000 µg/L
Pocillopora damicornis Calicoblast Cells – light 4 hr	10 µg/L	100 µg/L
Pocillopora damicornis Gastrodermal Cells – light 4 hr	1,000 µg/L	10,000 µg/L
Porites lobata Calicoblast Cells – light 4 hr	ND	ND
Symbiodinium Clade B from ***Pocillopora damicornis*** – 96 hr Growth Inhibition	>15,000 µg/L	>15,000 µg/L
Symbiodinium Clade B from ***Pocillopora damicornis*** – 96 hr Photosynthetic Efficiency (YII)	>15,000 µg/L	>15,000 µg/L

Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine



HMX

Background

HMX (CAS # 2691-41-0; IUPAC, Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine; other synonyms: Cyclotetramethylenetetranitramine, Octogen, 1,3,5,7-tetranitro-1,3,5,7-tetraazacyclooctane; MW: 296.20; color/form: white/crystals) is used in the explosives industry as propellants (base charge detonators and bursting-charge) and plastic explosives. This compound enters the aquatic environment through the disposal of raw materials from the manufacturing process and the corrosion of unexploded ordinance. Seawater solubility has been reported as 2 mg/L (Lutufo et al. 2010). The EC_{50} value for fresh water or marine algae has been reported to be >32 mg/L (Environmental Laboratory 1998; Lotufo et al. 2010). Other ecotoxicity values were reported for marine amphipod and polychaete with 115 mg/Kg and 353 mg/Kg NOEC values, respectively (Lotufo et al. 2001).

Coral Cell Toxicity Testing

An eight-point concentration gradient from 1 µg/L to 100,000 µg/L of HMX was used for the exposures of calicoblast and gastrodermal cells from *Porites divaricata* and *Pocillopora damicornis.* These concentrations exceeded the solubility of this compound in seawater, yet low levels of toxicity were detected at the higher concentrations. Primary cell cultures were isolated and cultured as described in the methods and plated into 24-well Teflon® plates at approximately $6.5x10^5$ to $1x10^6$ cells per well. Cells were exposed for 4 h in the light (295 µmol $m^{-2}s^{-1}$) at 25°C. Cell viability counts were performed on each well using the trypan blue exclusion dye viability assay as the endpoint for effects characterization.

Observed-effect concentrations (NOEC and LOEC)

Exposure of ***Porites divaricata*** **calicoblast** cells to various concentrations of HMX showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparisons test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was 1,000 µg/L (1 ppm) and the LOEC was 10,000 µg/L (10 ppm) (Fig. 50A; Table 20).

***Porites divaricata* gastrodermal** cells showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparisons test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC 1,000 µg/L (1 ppm) and the LOEC was 10,000 µg/L (10 ppm) (Fig. 50B; Table 20).

Exposure of ***Pocillopora damicornis* calicoblast** cells to various concentrations of HMX showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparisons test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was 1,000 µg/L (1 ppm) and the LOEC was 10,000 µg/L (10 ppm) (Fig. 50C; Table 20).

***Pocillopora damicornis* gastrodermal** cells showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparison test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was 10 µg/L (10 ppb) and the LOEC was 100 µg/L (100 ppb) (Fig. 50D; Table 20).

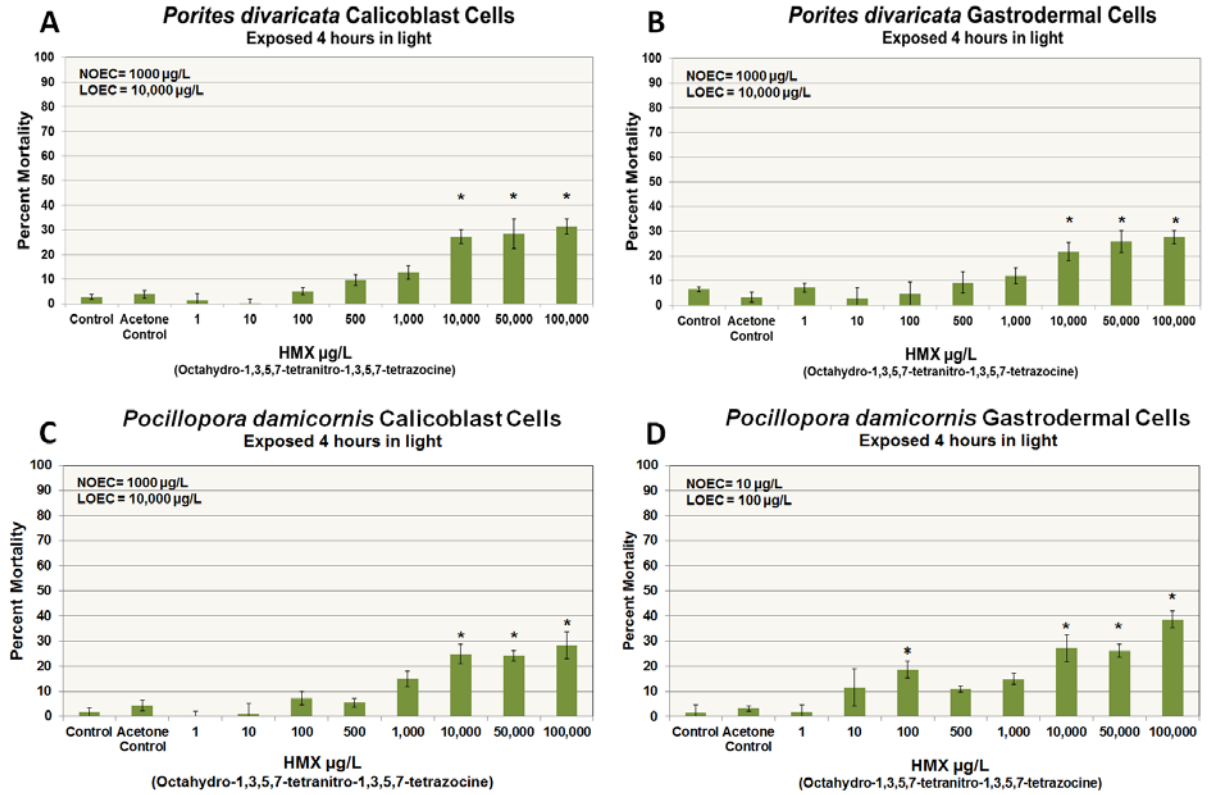


Figure 50. Percent mortality of *Porites divaricata* and *Pocillopora damicornis* cells exposed to various concentrations of HMX. n=4 replicates per treatment. Bars show treatment means with ±1 SEM. Treatment means with * differed significantly at $\alpha = 0.05$, one-way ANOVA followed by Dunnett's test for multiple comparisons test versus the carrier control. A) *P. divaricata* calicoblast cells. B) *P. divaricata* gastrodermal cells. C) *P. damicornis* calicoblast cells. D) *P. damicornis* gastrodermal cells. NOEC=no-observed-effect concentration. LOEC=lowest-observed effect concentration.

Symbiodinium sp. Clade B Culture Toxicity Testing

Symbiodinium sp. Clade B was not tested with HMX.

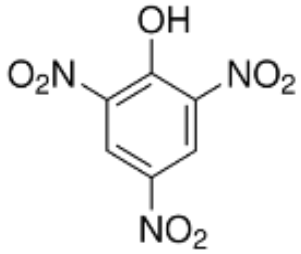
Summary of Toxicity Testing of HMX

Summary reference toxicity values are presented in the following two tables. Table 20 provides summary information for NOEC and LOEC values that were statistically significant ($P<0.05$) different from control treatments. No effect concentration values for either lethality (LC) or sub-lethal effects (EC) could be determined for HMX at the concentrations tested.

Table 20. Summary of No-observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) for all experiments exposing coral specimens to HMX. ND=Not Determined.

Test Organism	NOEC HMX	LOEC HMX
Porites divaricata Calicoblast Cells – light 4 hr	1000 µg/L	10,000 µg/L
Porites divaricata Gastrodermal Cells – light 4 hr	1000 µg/L	10,000 µg/L
Pocillopora damicornis Calicoblast Cells – light 4 hr	1000 µg/L	10,000 µg/L
Pocillopora damicornis Gastrodermal Cells – light 4 hr	10 µg/L	100 µg/L
Porites lobata Calicoblast Cells – light 4 hr	ND	ND
Symbiodinium Clade B from ***Pocillopora damicornis*** – 96 hr Growth Inhibition	ND	ND
Symbiodinium Clade B from ***Pocillopora damicornis*** – 96 hr Photosynthetic Efficiency (YII)	ND	ND

2,4,6-Trinitrophenol



Picric Acid

Background

Picric Acid (CAS # 88-89-1; IUPAC, 2,4,6-trinitrophenol, other synonyms: 2-hydroxy-1,3,5-trinitrobenzene; carbazotic acid, phenol trinitrate, picronitric acid; MW 229.10 g/mol; color/form: yellow/crystals) Picric acid is most commonly used in various histological dyes and fixatives, (e.g., Bouin's fixative). However later, it was recognized that the acid as well as the salts of picric acid could be used as an explosive. It is extremely unstable and explosive when dry, crystalline form, but the acid can also serve as an explosive with the aid of detonation. This highly nitrated compound has been used since the 1800's in the explosives industry and can be found in rocket fuel. Its solubility in water is 12.7 g/L. Review of the literature (Nipper et al. 2001; Nipper et al. 2002; Carr 2003) indicated 101-138 mg/L range of EC_{50} values for freshwater or marine algae germling cell number and 267-296 mg/L range of EC_{50} values for *Arbacia punctulata,* sea urchin embryo development.

Coral Cell Toxicity Testing

An eight-point concentration gradient from 1 to 500,000 µg/L of picric acid was used for the exposures of calicoblast and gastrodermal cells from *Porites divaricata* and *Pocillopora damicornis*. In this series of experiments the picric acid was not pH buffered, but allowed to reach its pH based on the buffering capacity of 35 ppt seawater-containing culture media. Primary cells were isolated and cultured as described in the methods and plated into 24-well Teflon® plates at approximately $6.5x10^5$ to $1x10^6$ cells per well. Cells were exposed for 4 h in the light (295 µmol $m^{-2}s^{-1}$) at 25°C. Cell viability counts were performed on each well using the trypan blue exclusion dye viability assay as the endpoint for effects characterization.

Observed-effect concentrations (NOEC and LOEC)

Exposure of ***Porites divaricata*** **calicoblast** cells to various concentrations of picric acid showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparisons test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was 1 µg/L (1 ppb) and the LOEC was 10 µg/L (10 ppb) (Fig. 51A; Table 21).

Porites divaricata **gastrodermal** cells showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc

Dunnett's multiple comparisons test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was <1 µg/L (<1 ppb) and the LOEC was 1 µg/L (1 ppb) (Fig. 51B; Table 21).

Exposure of ***Pocillopora damicornis*** **calicoblast** cells to various concentrations of picric acid showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparisons test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was <1 µg/L (<1 ppb) and the LOEC was 1 µg/L (1 ppb) (Fig. 51C; Table 21).

Pocillopora damicornis **gastrodermal** cells showed a significant effect on percent mortality ($p<0.0001$) when compared to controls as determined by a one-way ANOVA. The post-hoc Dunnett's multiple comparisons test found significant differences ($p<0.05$) among treatments versus the control group. The NOEC was 10 µg/L (10 ppb) and the LOEC was 100 µg/L (100 ppb) (Fig. 51D; Table 21).

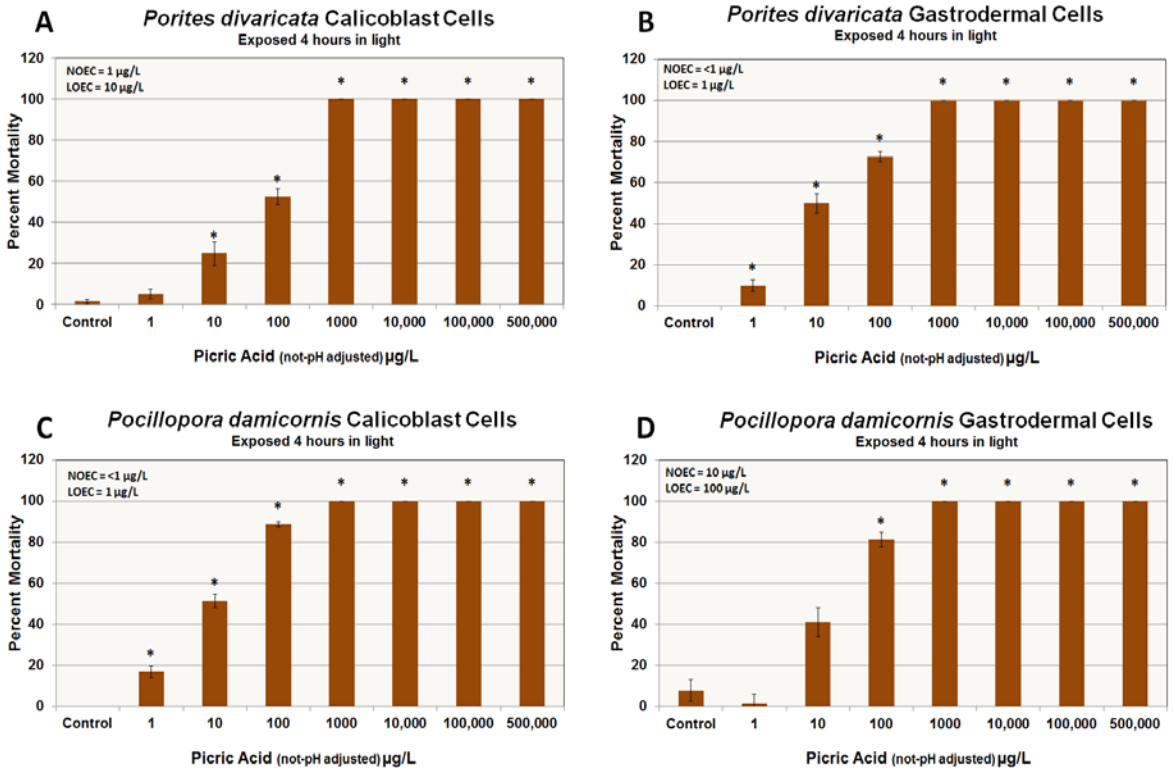


Figure 51. Percent mortality of *Porites divaricata* and *Pocillopora damicornis* cells exposed to various concentrations of picric acid. n=4 replicates per treatment. Bars show treatment means with ±1 SEM. Treatment means with * differed significantly at $\alpha = 0.05$, one-way ANOVA followed by Dunnett's test for multiple comparisons versus the carrier control. A) *P. divaricata* calicoblast cells. B) *P. divaricata* gastrodermal cells. C) *P. damicornis* calicoblast cells. D) *P. damicornis* gastrodermal cells. NOEC=no-observed-effect concentration. LOEC=lowest-observed effect concentration.

LC_{50} and LC_{20}

Porities divaricata and *Pocillopora damicornis* calicoblast and gastrodermal cell mortality data for picric acid exposures were subjected to PROBIT analysis to determine LC_{50} and LC_{20} values and confidence intervals.

The LC_{50} for ***Porites divaricata*** **calicoblast cells** was 60.4 µg/L (60.4 ppm) and the LC_{20} was 10 µg/L (10 ppb) (Fig. 52A; Table 22). The LC_{50} for ***Porites divaricata*** **gastrodermal** cells was 20.5 µg/L (20.5 ppb) and the LC_{20} was 2.1 µg/L (2.1 ppb) (Fig. 52B; Table 22).

The LC_{50} for ***Pocillopora damicornis*** **calicoblast** cells was 10.5 µg/L (10.5 ppb) and the LC_{20} was 1.2 µg/L (1.2 ppb) (Fig. 52C; Table 22). The LC_{50} of ***Pocillopora damicornis*** **gastrodermal** cells was 22 µg/L (22 ppb) and the LC_{20} was 3.7 µg/L (3.7 ppb) (Fig. 52D; Table 22).

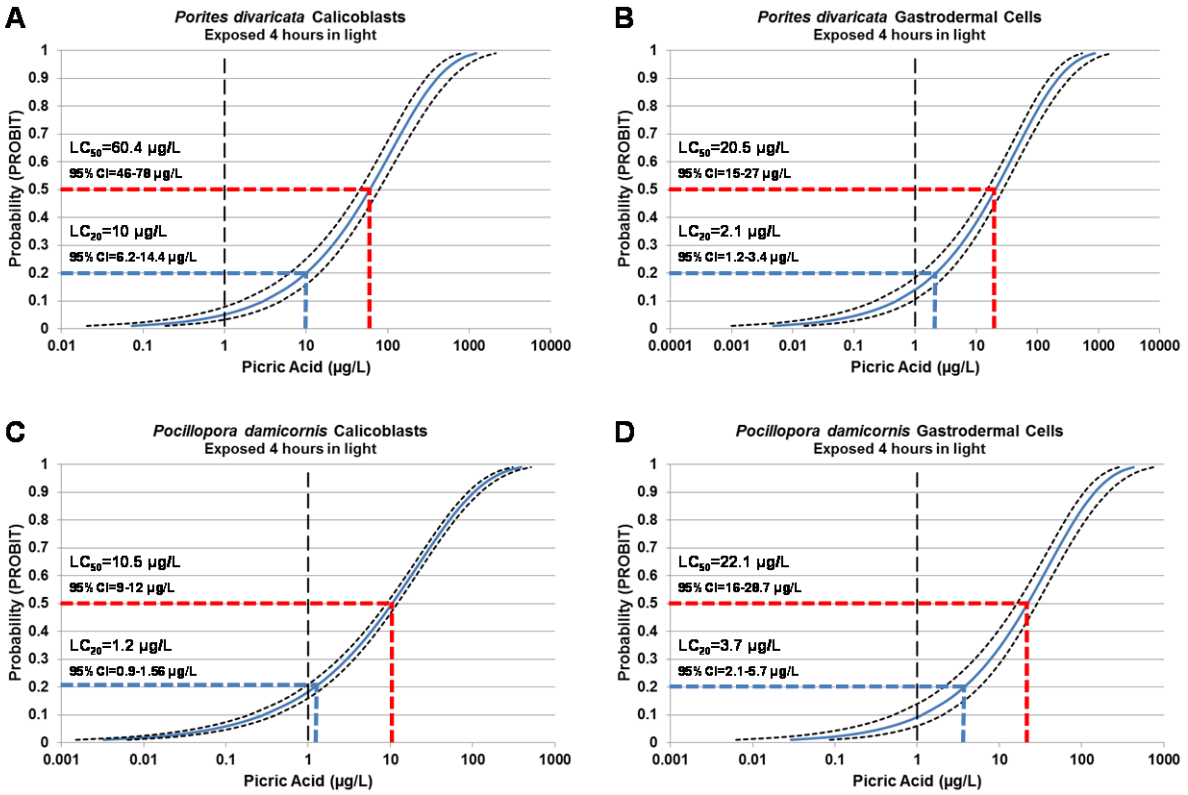


Figure 52. Results of PROBIT analyses LC_{50} and LC_{20} values for coral cells exposed to picric acid. A) *P. divaricata* calicoblast cells. B) *P. divaricata* gastrodermal cells. C) *P. damicornis* calicoblast cells. D) *P. damicornis* gastrodermal cells. Vertical bars indicate dose range tested.

Symbiodinium sp. Clade B Culture Toxicity Testing

Symbiodinium sp. Clade B was not tested with picric acid.

Summary of Toxicity Testing of Picric Acid

Summary reference toxicity values are presented in the following two tables. Table 21 provides summary information for NOEC and LOEC values that were statistically significant ($P<0.05$) different from control treatments. Table 22 provides summary information for effect concentration values for either lethality (LC) or sub-lethal effects (EC) for all testing of coral for picric acid.

Table 21. Summary of No-observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) for all experiments exposing coral specimens to unbuffered Picric Acid. ND=Not Determined.

Test Organism	NOEC Picric Acid	LOEC Picric Acid
Porites divaricata Calicoblast Cells – light 4 hr	1 µg/L	10 µg/L
Porites divaricata Gastrodermal Cells – light 4 hr	<1 µg/L	1 µg/L
Pocillopora damicornis Calicoblast Cells – light 4 hr	<1 µg/L	1 µg/L
Pocillopora damicornis Gastrodermal Cells – light 4 hr	10 µg/L	100 µg/L
Symbiodinium Clade B from *Pocillopora damicornis* – 96 hr Growth Inhibition	ND	ND
Symbiodinium Clade B from *Pocillopora damicornis* – 96 hr Photosynthetic Efficiency (YII)	ND	ND
Porites divaricata Fragment Total Porphyrin – 96 hr	ND	ND

Table 22. Summary of lethal concentrations ($LC_{50,}$ LC_{20}) and effect concentrations (EC_{50}, EC_{20}) for all experiments exposing coral specimens to picric acid. ND=Not Determined.

Test Organism	LC_{50} Picric Acid	LC_{20} Picric Acid
Porites divaricata **Calicoblast Cells – light 4 h**	**60.4 µg/L** 95% CI = 46-78 µg/L	**10 µg/L** 95% CI = 6.2-14.4 µg/L
Porites divaricata **Gastrodermal Cells – light 4 h**	**20.5 µg/L** 95% CI =15-27 µg/L	**2.1 µg/L** 95% CI = 1.2-3.4 µg/L
Pocillopora damicornis **Calicoblast Cells – light 4 h**	**10.5 µg/L** 95% CI = 9-12 µg/L	**1.2 µg/L** 95% CI = 0.9-1.56 µg/L
Pocillopora damicornis **Gastrodermal Cells – light 4 h**	**22 µg/L** 95% CI = 16-28.7 µg/L	**3.7 µg/L** 95% CI = 2.1-5.7 µg/L
	EC_{50}	EC_{20}
Symbiodinium Clade B – 96 h ***Pocillopora damicornis*** **Growth Inhibition**	**ND**	**ND**
Symbiodinium Clade B - 96 h ***Pocillopora damicornis*** **Photosynthetic Efficiency (YII)**	**ND**	**ND**
Porites divaricata **Fragment Total Porphyrin – 96 h**	**ND**	**ND**

Conclusions

Summary of Findings for Task 1: Laboratory-based Toxicity Testing of MCs

1.1 Are MCs toxic to coral?

1.2 What are the concentration ranges of toxic effects?

1. What is the relative toxicity of munitions compounds to coral species and coral cell type?

Munitions compounds tested in coral cell toxicity assays included three nitrotoluene compounds: TNT and two of its major breakdown products, 2,4-DNT and 2,6-DNT; two nitramines, RDX and HMX; and one nitrophenol, picric acid (2,4,6-trinitrophenol). Based on comparisons of LC_{50} values, picric acid was consistently the most toxic (lowest LC_{50}) of the compounds tested, except for *Pocillopora damicornis* gastrodermal cells exposed in light conditions in which TNT had the lowest LC_{50} value. In this case the LC_{50} values were very close (15.3 vs 22.1 µg/L), thus reversing their rank order positions. The nitrotoluenes ranked next with TNT being the most toxic followed by 2,4-DNT and 2,6-DNT being the least toxic of the three compounds.

Though some of the highest treatments of RDX (up to 10,000 µg/L (10 ppm)) (Fig. 45) and HMX (up to 100,000 µg/L (100 ppm)) (Fig. 50) were found to be significantly ($p<0.05$) different from control treatments, median lethal concentrations could not be determined by PROBIT models for either RDX or HMX. This is because the effect (percent mortality) did not reach the 50% threshold required by PROBIT models to estimate LC_{50} values. RDX was tested at concentrations as high as 10,000 µg/L (10 ppm) with only 25-30% mortality achieved in the cell toxicity assay. HMX was tested at concentrations as high as 100,000 µg/L (100 ppm). Even though this is above its reported solubility in seawater, a 30-35% level of mortality was achieved. This level was insufficient for calculating median lethal effect concentration, thus toxicities of RDX and HMX could not be ranked. It does appear, however, that RDX and HMX are far less toxic than the other MCs tested in these bioassays. Summaries of NOEC, LOEC can be found in Table 23 and EC_{50} and EC_{20} in Table 24.

- The relative effect of munitions compounds tested on ***Porites divaricata*** **calicoblast** cells, 4 hours in **light** endpoint:

Mortality LC_{50}:

	Picric acid	►	**2,4,6-TNT**	►	**2,4-DNT**	►	**2,6-DNT**	**? RDX**	**?HMX**
(µg/L)	**60.4**	►	**716**	►	**5,818**	►	**16,557**	**?**	**?**

- The relative effect of munitions compounds tested on ***Porites divaricata*** **gastrodermal** cells, 4 hours in **light** endpoint:

Mortality LC_{50}:

	Picric acid	►	**2,4,6-TNT**	►	**2,4-DNT**	►	**2,6-DNT**	**? RDX**	**?HMX**
(µg/L)	**20.5**	►	**54**	►	**3,123**	►	**3,699**	**?**	**?**

- The relative effect of munitions compounds tested on ***Pocillopora damicornis*** **calicoblast** cells, 4 hours in **light** endpoint:

Mortality LC_{50}:

	Picric acid	►	**2,4,6-TNT**	►	**2,4-DNT**	►	**2,6-DNT**	≈	**? RDX**		**?HMX**
(µg/L)	**10.5**	►	**16**	►	**1,284**	►	**11,075**	≈	**?**		**?**

- The relative effect of munitions compounds tested on ***Pocillopora damicornis*** **gastrodermal** cells, 4 hours in **light** endpoint:

Mortality LC_{50}:

	2,4,6-TNT	►	**Picric acid**	►	**2,4-DNT**	►	**2,6-DNT**	≈	**? RDX**		**?HMX**
(µg/L)	**15.3**	►	**22.1**	►	**565**	►	**1,844**	≈	**?**		**?**

2. Does light affect ***Porites divaricata*** *coral cell types differentially when they are exposed to varying concentrations of TNT?*

Early observations with exposures of *Pocillopora damicornis* cells to varying concentrations of TNT showed anomalous behaviors of calicoblast cells when attempting to stain them with vital dyes (to determine live-dead condition). To assess mortality it became necessary to use cell counts that were plated initially, subtracting cells remaining at the end of the exposure. *Pocillopora damicornis* gastrodermal cells exposed to concentrations of TNT >100 µg/L in the dark would quickly rupture once subjected to bright-field microscopy for vital stain assessment. These observations led to investigating whether light exposure resulted in photo-induced or photo-enhanced toxicity of TNT with another species and cell type. The results of exposing *Porites divaricata* calicoblast and gastrodermal cells indicated there is a difference; however, the response is more complex than expected. *Porites divaricata* gastrodermal cells were most sensitive to TNT exposure, if they were exposed for 4 h in the light vs exposure in the dark. In fact, they were approximately 20 times more sensitive to TNT when exposed in the light. The *P. divaracata* calicoblast responses to exposure to TNT in the dark vs light were very similar, with slightly more sensitivity for cells exposed in the light vs dark conditions. Comparisons of *P. damicornis* cells exposed in light and dark conditions, showed the calicoblasts of this species were more sensitive to TNT than their gastrodermal cells when exposed in the light vs dark conditions.

- The relative effect of TNT exposure under light and dark conditions on ***Porites divaricata*** **calicoblast (CB) and gastrodermal (GD)** cells:

Mortality LC_{50}:

	GD light	►	**CB light**	►	**CB dark**	►	**GD dark**
(µg/L)	**54**	►	**716**	►	**968**	►	**1,196**

- The relative effect of TNT exposure under light and dark conditions on ***Pocillopora damicornis*** **calicoblast (CB) and gastrodermal (GD)** cells:

Mortality LC_{50}:

	GD light	►	CB light	►	GD dark	►	CB dark
(µg/L)	**15.3**	**►**	**16**	**►**	**140**	**►**	**1,582**

3. Is there a difference in response to MCs based on coral species or coral cell type?

Three species and two cell types from each were tested to determine if they exhibited differential sensitivity among the species and cell types to 2,6-DNT exposure in light conditions. The results show very markedly a difference in species response as well as a segregation of cell type responses that correspond to each species' relative sensitivity to 2,6-DNT exposure. *Pocillopora damicornis* was the most sensitive of the three species, followed by *Porites divaricata* and the least affected by this compound was *Porites lobata*. It is of interest to note that *P. lobata* is a mounding coral while the other two are branching species. The relevance of mounding vs branching species to susceptibility to MC exposure could not be determined by these laboratory exposure experiments. However these findings are the first to use an *in vitro* coral cell-based toxicity test for dose-response characterization and comparisons among these three shallow-water coral species to 2,6-DNT. Finding differing responses among the three species is important because this begins to lay a foundation for predicting possible ecological impacts and risk that may differ across sites depending on the species present at any particular reef site.

- The relative effect of 2,6-DNT exposure to ***Porites lobata (Plob), Porites divaricata(Pdiv) and Pocillopora damicornis(Pdam)*** **calicoblast (CB or gastrodermal cells (GD)**:

Mortality LC50:

	***Pdam* GD**	►	***Pdiv* GD**	►	***Plob* GD**	►	***Pdam* CB**	►	***Pdiv* CB**	►	***Plob* CB**
(µg/L)	**1,844**	**►**	**3,699**	**►**	**4,748**	**►**	**11,075**	**►**	**16,557**	**►**	**105,124**

4. What is the relative effect of munitions compounds on the coral endosymbiotic dinoflagellate, Symbiodinium?

Munitions compounds tested in static 96 h *Symbiodinium* sp. Clade B cell culture toxicity assays included six nitrotoluene compounds: TNT, the parent compound, three dinitrotoluene compounds, including two of its major breakdown products, 2,4-DNT and 2,6-DNT and a minor isomer 2,3-DNT; one nitrotoluene, 4-NT; one aminodinitrotoluene, 2-ADNT; and one nitramine, RDX. Comparisons of relative toxicity among these MCs was evaluated with two physiological endpoints: cell growth and photosynthetic efficiency. Both endpoints gave similar rankings for

relative toxicities; however, TNT's relative position in toxicity changed depending on the physiological endpoint. Kautsky light curve analyses that captured information on Y(II), Y(NO) and Y(NPQ) provided some information about how these compounds were affecting photosystem II performance. From these data, the mechanism of action appears to differ between compounds, as they affect cultured *Symbiodinium* sp. cells. For example, there is a common trend between 2,4-DNT, 2,6-DNT and 2-ADNT but a different response than seen with TNT or 2,3-DNT. In addition, because this analysis recorded response over time, this too varied with individual compounds. [see Appendix IV for graphs of individual compounds]

It should be noted that chemical analysis of the spent culture media indicated a breakdown in TNT over the course of the 96 h exposure (media was not replenished). Therefore, the response of the *Symbiodinium* cells was complicated by a buildup of toxic TNT breakdown products, (primarily 2,4-DNT and 2,6-DNT). Therefore to determine the toxicity of TNT alone would require treatment medium replenishment every 12 h, at a minimum. During this experiment, sterile (abiotic) media controls were also incubated along with the *Symbiodinium* cell cultures under in the same experimental set-up. Chemical analysis of the abiotic samples indicated that TNT was not breaking down due to the physical conditions (i.e., lighting, media, temperature). We hypothesize that the breakdown was due to either metabolism by the *Symbiodinium* cells or to microorganisms associated with the non-axenic culture of this dinoflagellate (axenic dinoflagellate cultures are rarely possible because of a suspected need for microbe interactions that make these *in vitro* cultures possible).

Comparisons of EC_{50} values based on *Symbiodinium* sp. cell growth ranked TNT as the most toxic and 4-NT the least toxic of the nitrotoluenes. This is consistent with the literature which indicates toxicity varies with the number and position of nitro groups. RDX exposures had no significant effect on cell growth at any concentration tested up to 15,000 µg/L, which approaches its solubility in seawater (19,770 µg/L at 25°C). Effects on photosynthetic efficiency were similar to those for growth; however 2,3-DNT appeared to cause the most significant effects on this parameter. RDX had no effect on photosynthetic efficiency (Table 24).

- The relative effect of munitions compounds tested on the symbiotic dinoflagellate, *Symbiodinium* sp., 96 h endpoint:

EC_{50} Cell Growth

2,4,6-TNT	►	**2,4-DNT**	►	**2,3-DNT**	►	**2-ADNT**	►	**2,6-DNT**	►	**4-NT**	≈	**?RDX**
(µg/L) 544	►	**1,315**	►	**2,524**	►	**4,059**	►	**22,292**	►	**116,083**	≈	**?**

EC_{50} Photosynthetic efficiency:

2,3-DNT	►	**2,4-DNT**	►	**2,4,6-TNT**	►	**2-ADNT**	►	**2,6-DNT**	►	**4-NT**	≈	**?RDX**
(µg/L) 2,810	►	**4,814**	►	**7,039**	►	**10,206**	►	**45,516**	►	**137,902**	≈	**?**

5. Do cell-based assays reflect toxicity of intact corals?

Our initial project tasks called for exposure-response studies for intact coral fragments after conducting field studies to characterize exposure and biological effects. However, based on concerns expressed by the IPR Board regarding the whether cell-based and intact coral fragment-based assays correlated, we conducted 96 h exposures for three MCs using intact coral fragments. Though 96 h exposures of whole organisms are not direct comparisons with the 4 h cell-based assays, findings were consistent across endpoints. Our findings from these preliminary experiments are as follow:

- RDX showed no toxic response in *Pocillopora damicornis* fragments as assessed by endpoints that include visual inspection of changes in polyp behavior, tissue integrity or color; photosynthetic efficiency as measured by PAM fluorometry; changes in tissue morphology as assessed by histopathology; or in cellular physiological endpoints of total porphyrin or DNA damage as assessed by DNA AP site numbers. No significant effects were observed in cultures of the coral symbiotic dinoflagellate, *Symbiodinium* sp. *Pocillopora damicornis* or *Porites divaricata* calicoblast cells which showed 10-20% mortality at doses above 100 µg/L, while gastrodermal cell were 10-100 times more tolerant of this compound. The highest concentration used in the fragment exposures was 16,000 µg/L for comparison.

- 2,3-DNT showed signs of lethal toxicity in *Pocillopora damicornis* with severe tissue loss, at concentrations of 2,000 µg/L and higher within 18 h of exposure. This was unexpected based on the previous *Symbiodinium* testing. A second exposure was conducted with sub-lethal concentrations which showed significant ($p<0.05$) depression in total porphyrin levels (an indicator of metabolic dysfunction) at 292 µg/L and higher concentrations. Unfortunately coral cell toxicity testing was not conducted to allow comparison, though we expect coral cells would show higher sensitivity.

- TNT showed significant toxic effects in *Porites divaricata* fragments. Cell toxicity assays showed significant ($p<0.05$) effects across a range of TNT concentrations, overlapping those used for the fragment dosing. Significant ($p<0.05$) changes at concentrations of 100 µg/L and higher were documented in visual physio-scoring of changes in polyp behavior, tissue integrity and necrosis. Histopathological changes were also seen along a gradient of concentrations with severe tissue disruption and necrosis observed at the 25,000 µg/L concentration (50,000 µg/L treatment not analyzed because of catastrophic tissue loss). TEM confirmed ultrastructural changes were also occurring, affecting gastrodermal and epidermal cells. Cellular physiological measures showed a depression in total porphyrin levels at 2,500 µg/L TNT following 24 h of exposure, indicating generalized metabolic disruption. Adverse effects on *Symbiodinium* cell growth and photosynthetic efficiency were found at concentrations as low as 250 µg/L TNT.

- An important and frequently asked question is how well do *in vitro cell* toxicity assays reflect responses in intact coral? Realizing coral cells are much more sensitive to toxicants than intact organisms, a correction factor is necessary to translate coral cell mortality into potential mortality of coral fragments. To address this question a regression analysis was conducted using *Porites divaricata* calicoblast and gastrodermal cells (both in light and dark

exposure conditions) and intact *P. divaricata* fragments exposed for 96 h to TNT. Strong positive relationships existed when *in vitro* cell mortalities were regressed against coral fragment necrosis for TNT exposed *Porites divaricata*. Thus, *in vitro* cell mortality is a potential indicator of coral fragment necrosis. To estimate the correction factor needed to translate coral cell mortality into potential necrosis of coral fragments (e.g., coral death), a polynomial regression model can be used. Each of the following regression models performed very well in every case ($p < 0.05$, $r^2 > 0.95$), and each followed the normal distribution (Shapiro-Wilk, $p > 0.05$). Any one of these models may be used to successfully predict coral fragment necrosis:

For gastrodermis cells in the light ($F_{2,7} = 81.32$, $p = 0.0002$, $r^2 = 0.97$):
% Necrosis of fragments = 18.0
- 1.17 (% Mortality of cells)
+ 0.0190 (% Mortality of cells)^2

For gastrodermis cells in the dark ($F_{2,7} = 63.36$, $p = 0.0003$, $r^2 = 0.96$):
% Necrosis of fragments = -1.69
+ 0.138 (% Mortality of cells)
+ 0.0116 (% Mortality of cells)^2

For calicoblast cells in the dark ($F_{2,7} = 152.0$, $p < 0.0001$, $r^2 = 0.98$):
% Necrosis of fragments = -2.81
+ 0.184 (% Mortality of cells)
+ 0.00965 (% Mortality of cells)^2

For calicoblast cells in the light ($F_{2,7} = 44.36$, $p = 0.0007$, $r^2 = 0.95$):
% Necrosis of fragments = 3.53
- 0.140 (% Mortality of cells)
+ 0.00989 (% Mortality of cells)^2

Evaluation of Results to Date Relative to the Go/No-Go Decision Point

We have successfully completed Task 1: Laboratory-based toxicity testing of MCs. We have determined that all nine munitions compounds have some level of toxicity in one or more of the bioassays conducted**.**

While static laboratory experiments are a good indicator for initial toxicity studies, often they do not accurately reflect the environmental conditions at field sites where coral and munitions are found together. We are yet to be able to understand how corals react to these compounds *in situ*, i.e., on the reef, under environmentally relevant conditions (chemical, physical and biological). To understand the environmental relevance of these laboratory findings requires moving from laboratory toxicity testing to field studies. The goal of this project has always been to conduct an ecological risk assessment (ERA) (Tasks 2-5). It is the field studies coupled with laboratory analyses of field samples that allow determination of environmentally relevant concentrations

and identity of compounds (exposure characterization). Bioassays of coral tissues from the environment help characterize the nature and severity of biological effects under environmentally relevant conditions (chemical, physical and biological influencing factors). Once these parameters are known, then validating the field work with targeted exposure characterizations with key compounds at environmentally relevant concentrations will decrease uncertainty and provide the means for developing a robust risk characterization model for the effects of munitions compounds on coral and coral reef health.

The objectives in Tasks 2-5 were proposed to address these unknowns and to synthesize the information into an ecological risk model for coral reef munitions exposure. We have evidence that select munitions compounds harm coral and believe that follow-up field studies are justified.

Table 23. Summary of No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) Across All Experiments

Species	Biological Endpoint	Exposure Condition	Exposure Duration	Statistical Endpoint	Compound Tested (µg/L)								
					TNT	2,3 DNT	2,4 DNT	2,6 DNT	4-NT	2-ADNT	RDX	HMX	Picric Acid
Porites lobata	Calicoblast	Light	4 h	NOEC				500					
				LOEC				2,500					
Porites lobata	Gastrodermal	Light	4 h	NOEC				25					
				LOEC				100					
Porites divaricata	Calicoblast	Light	4 h	NOEC	100		0.5	25			100	1,000	1
				LOEC	500		5	100			500	10,000	10
Porites divaricata	Calicoblast	Dark	4 h	NOEC	100								
				LOEC	500								
Porites divaricata	Gastrodermal	Light	4 h	NOEC	25		25	5			500	1,000	<1
				LOEC	100		100	25			1,000	10,000	1
Porites divaricata	Gastrodermal	Dark	4 h	NOEC	25								
				LOEC	100								
Porites divaricata	Fragment Porphyrin	Diurnal 16:8 D:L	24 h	NOEC	500								
				LOEC	2,500								
Porites divaricata	Fragment Porphyrin	Diurnal 16:8 D:L	96 h	NOEC	500								
				LOEC	2,500								
Pocillopora damicornis	Calicoblast	Light	4 h	NOEC	<0.5		5	100			10	1,000	<1
				LOEC	0.5		25	500			100	10,000	1
Pocillopora damicornis	Calicoblast	Dark	4 h	NOEC	25								
				LOEC	100								
Pocillopora damicornis	Gastrodermal	Light	4 h	NOEC	0.5		0.1	5			1,000	10	10
				LOEC	5		0.5	25			10,000	100	100
Pocillopora damicornis	Gastrodermal	Dark	4 h	NOEC	0.5								
				LOEC	5								
Pocillopora damicornis	Fragment Porphyrin	Diurnal 16:8 D:L	96 h	NOEC		162					>16,000		
				LOEC		292					>16,000		
Symbiodinium Clade B	Dinoflagellate Growth	Diurnal	96 h	NOEC	<250	1,250	310	12,500	25,000	630	>15,000		
				LOEC	250	2,500	630	25,000	50,000	1,250	>15,000		
Symbiodinium Clade B	Dinoflagellate Photosyn. Effic.	Diurnal	96 h	NOEC	<250	<160	630	12,500	25,000	<630	>15,000		
				LOEC	250	160	1,250	25,000	50,000	630	>15,000		

Table 24. Summary of Lethal (LC) and Effect (EC) Concentrations Across All Experiments

Species	Biological Endpoint	Exposure Condition	Exposure Duration	Statistical Endpoint	Compound Tested (µg/L)								
					TNT	2,3 DNT	2,4 DNT	2,6 DNT	4-NT	2-ADNT	RDX	HMX	Picric Acid
Porites lobata	Calicoblast	Light	4 h	LC_{50}				105,124					
				LC_{20}				948					
Porites lobata	Gastrodermal	Light	4 h	LC_{50}				4,748					
				LC_{20}				216					
Porites divaricata	Calicoblast	Light	4 h	LC_{50}	716		5,818	16,557			DMF	DMF	60.4
				LC_{20}	60.5		79	631			DMF	DMF	10
Porites divaricata	Calicoblast	Dark	4 h	LC_{50}	968								
				LC_{20}	32.6								
Porites divaricata	Gastrodermal	Light	4 h	LC_{50}	54		3,123	3,699			DMF	DMF	20.5
				LC_{20}	0.36		52	137			DMF	DMF	2.1
Porites divaricata	Gastrodermal	Dark	4 h	LC_{50}	1,196								
				LC_{20}	21								
Porites divaricata	Fragment Porphyrin	Diurnal 16:8 D:L	24 h	EC_{50}	5,416								
				EC_{20}	DMF								
Porites divaricata	Fragment Porphyrin	Diurnal 16:8 D:L	96 h	EC_{50}	DMF								
				EC_{20}	DMF								
Pocillopora damicornis	Calicoblast	Light	4 h	LC_{50}	16		1,284	11,075			DMF	DMF	10.5
				LC_{20}	1.85		15	463			DMF	DMF	1.2
Pocillopora damicornis	Calicoblast	Dark	4 h	LC_{50}	1582								
				LC_{20}	200								
Pocillopora damicornis	Gastrodermal	Light	4 h	LC_{50}	15.3		565	1,844			DMF	DMF	22.1
				LC_{20}	2.15		2	71			DMF	DMF	3.7
Pocillopora damicornis	Gastrodermal	Dark	4 h	LC_{50}	140								
				LC_{20}	12.7								
Pocillopora damicornis	Fragment Porphyrin	Diurnal 16:8 D:L	96 h	EC_{50}		DMF							
				EC_{20}		DMF							
Symbiodinium Clade B	Dinoflagellate Growth	Diurnal	96 h	EC_{50}	544	2524	1,315	22,292	116,083	4,059	DMF		
				EC_{20}	17	1275	314	4,135	35,715	915	DMF		
Symbiodinium Clade B	Dinoflagellate Photosyn. Effic.	Diurnal	96 h	EC_{50}	7039	2810	4,814	45,516	137,902	10,206	DMF		
				EC_{20}	1908	2520	1,380	17,749	62,814	1,801	DMF		

DFM= Data Model Failed

Literature Cited

Asaeda A, Ide H, Terato H, Takamori Y, Kubo K (1998) Highly sensitive assay of DNA abasic sites in mammalian cells-optimization of the aldehyde reactive probe method. *Analytica Chimica Acta* 365: 35-41.

Avadanei, AR, May, LA, Woodley, CM (2012) *Porphyrin Detection in Denatured Cnidarian Tissue Extracts.* NOAA Technical Memorandum, NOS NCCOS 145 and CRCP 17. Silver Spring MD, National Oceanic and Atomospheric Administration. 44.

Boturyn D, Constant, JF, Defrancq E, Lhomme J, Barbin A, Wild CP (1999) A simple and sensitive method for *in vitro* quantitation of abasic sites in DNA. *Chem Res Toxicol* 12:476-482.

Carr, RS, Nipper, M (2003) *Assessment of environmental effects of ordnance compounds and their transformation products in coastal ecosystems.* Technical Report TR-2234-ENV. Naval Facilities Engineering Command, Port Hueneme, California. https://navfac.navy.mil/content/dam/navfac/Specialty%20Centers/Engineering%20and%20Expeditionary%20Warfare%20Center/Environmental/Restoration/er_pdfs/a/navfacesc-ev-tr-2234-env-ordcmpds-200312.pdf (Accessed January 2014)

Carson FL (1997) *Histotechnology: A Self-Instructional Text*. 2nd ed. American Society of Clinical Pathology Presss, Chicago, IL. 304 p.

Davy SK, Allemand D, Weis V (2012) Cell biology of cnidarian-dinoflagellate symbiosis. *Microbiol Molec Biol Rev* 76: 229-261.

Downs CA, Richmond RH, Mendiola WJ, Rougee L, Ostrander GK (2006) Cellular physiological effects of the MV Kyowa Violet fuel-oil spill on the hard coral, *Porites lobata. Environ Toxicol Chem* 25:3171-3180.

Downs CA, Fauth JF, Downs VD, Ostrander GK (2010) *In vitro* cell-toxicity screening as an alternative animal model for coral toxicology: effects of heat stress, sulfide, rotenone, cyanide, and cuprous oxide on cell viability and mitochondrial function. *Ecotoxicol* 19:171-184.

Downs CA, Woodley CM, Fauth J, Knutson S, Burtscher M, May LA, Avadanei AR, Higgins J, Ostrander G (2011) A survey of environmental pollutants and cellular-stress markers of Porites astreoides at six sites in St. John, U.S. Virgin Islands. *Ecotoxicol* 20: 1914-1931.

Downs CA, Kramarsky-Winter E, Fauth JE, Segal R, Bronstein O, Jeger R, Lichtenfeld Y, Woodley CM, Pennington P, Kushmaro A, Loya Y (2013) Toxicological effects of the sunscreen UV filter, benzophenone-2, on planulae and in vitro cells of the coral, *Stylophora pistillata. Ecotoxicol* [ahead of print] DOI: 10.1007/s10646-013-1161-y

Environmental Laboratory (1998) *Toxicity of military unique compounds in aquatic organisms: an annotated bibliography (studies published through 1996).* Technical Report IRRP-98-4. U.S. Army Engineer Waterways Experimental Station, Vicksburg, MS.

Hamilton M, Russo R, Thurston R (1977) Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. *Environ Sci Technol* 11: 714-719.

Karako-Lampert S, Katcoff DJ, Achituv Y, Dubinsky Z, Stambler N (2005) *J Exp Mar Biol Ecol* 318: 11-20.

Kholod YA, Gryn'ova G, Gorb L, Hill FC, Leszczynski J (2011) Evaluation of the dependence of aqueous solubility of nitro compounds on temperature and salinity: a COSMO-RS simulation. *Chemosphere* 83: 287-294.

Kinzie RA, Takayama M, Santos SR, Coffroth MA (2001) The adaptive bleaching hypothesis: experimental tests of critical assumptions. *Biol Bull* 200:51-58.

Lent EM, Crouse LCB, Quinn MJ, Wallace SM (2012) Assessment of the *in vivo* genotoxicity of isomers of dinitrotoluene using alkaline Comet and peripheral blood micronucleus assays. *Mutation Research/Genetic Toxicol Environ Mut* 742: 54-60.

Lotufo GR, Farrar D, Inouye LS, Bridges TS, Ringelberg DB (2001) Toxicity of sediment-associated nitroaromatic and cyclonitramine compounds to benthic invertebrates. *Environ Toxicol Chem* 20: 1762-1771.

Lotufo GR, Gibson AB, Yoo JL (2010) Toxicity and bioconcentration evaluation of RDX and HMX using sheepshead minnows in water exposures. *Ecotoxicol Environ Safe* 73: 1653-1657.

Martikainen EAT, Krogh PH (1999) Effects of soil organic matter content and temperature on toxicity of dimethoate to *Folsomia fimetaria* (Collembola: Isotomiidae). *Environ Toxicol Chem* 18: 865-872.

May LA (2011) *Chemiluminescenct DNA Abasic (AP) Site Assay*. CDHC Virtual Protocol 103_APSite. http://cdhc.noaa.gov/_docs/Virtual%20Chemiluminescent%20DNA%20AP%20Site%20Assay_formatted_11-15-11.pdf (Accessed Jan 2014).

Naval Facilities Engineering Command (2000) Development of marine sediment toxicity for ordnance compounds and toxicity identification evaluation studies at select naval facilities. Contract Report CR01-002-ENV. http://www.clu-in.org/download/contaminantfocus/sediments/tox_marine_sed.pdf (Accessed January 2014)

Newman M (1995) Quantitative Methods in Aquatic Ecotoxicology. In: *Advances in Trace Substances Research.* Lewis Publishers: Boca Raton, FL. pp.119-129.

Nipper M, Carr RS, Biedenbach JM, Hooten RL, Miller K (2002) Toxicological and chemical assessment of ordnance compounds in marine sediments and porewaters. *Mar Pollut Bull* 44: 789-806.

Nipper M, Carr RS, Biedenbach JM, Hooten RL, Miller K, Saepoff S (2001) Development of marine toxicity data for ordnance compounds. *Arch of Environ Contamin Toxicol.* 41: 308-318.

Nipper M, Carr RS, Lotufo GR (2009) Aquatic Toxicology of Explosives. In: *Ecotoxicology of Explosives* (ed: GI Sunahara, G Lutufo, RG Kuperman, J Hawari). CRC Press, Boca Raton, Florida. pp. 77-115.

Prak DJL, O'Sullivan DW (2007) Solubility of 4-nitrotoluene, 2,6-dinitrotoluene, 2,3-dinitrotoluene, and 1,3,5-trinitrobenzene in pure water and seawater. *J Chem Eng Data* 52: 2446-2450.

Prak DJL, O'Sullivan DW (2011) Assessing the salting-out behavior of 2,4-dinitrobenzaldehyde and 2,6-dinitrobenzaldehyde from solubility values in pure water and seawater at temperatures between (280 and 313) K. *J Chem Eng Data* 56: 2630-2633.

Ralph PJ, Schreiber U, Gademann R, Kuhl M, Larkum AWD (2005) Coral photobiology studied with a new imaging pulse amplitude modulated fluorometer. *J Phycol* 41: 335-342.

US Army Corps of Engineers, Environmental Laboratory (1998) "Toxicity of military unique compounds in aquatic organisms: An annotated bibliography (studies published through 1996)," Technical Report IRRP-98-4, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS. http://el.erdc.usace.army.mil/elpubs/pdf/trirrp98-4.pdf (Accessed January 2014)

Waisner S, Hansen L, Fredrickson H, Nestler C, Zappi M, Banerji S, Baipai R (2002) Biodegradation of RDX within soil-water slurries using a combination of differing redox incubation conditions. *J Hazard Mater* 95: 91-106.

Zhao Y, Yuan X, Guo J, Sheng L (1997) Quantitative structure-activity relationships of nitroaromatic compounds to four aquatic organisms. *Chemosphere* 34: 1837-1844.

Zar J (1999) *Biostatistical Analysis*. 4th ED. Prentice Hall Inc: Upper Saddle River, NJ. pp. 217-219.

Appendix I

Semi-Organic Extraction of Porphyrin from Coral Tissue

Adapted for use in stony corals by Thomas Bartlett, Laura Webster
September 17, 2013

Reagents Needed (see appendix for details)**:**

- **50 mM phosphate buffer, pH 8.0**:

  ~450 mL Milli-Q water
  3.3 g sodium phosphate dibasic, anhydrous
  0.24 g sodium phosphate monobasic, monohydrate.
  Adjust pH to 8.0 with 0.1 M NaOH. Bring volume up to 500 mL with Milli-Q.

- 100% acetonitrile (ACN)

- **Extraction buffer (EB)** = 25% acetonitrile in 50 mM phosphate buffer, pH 8.0:

  make fresh daily: 10 mLACN + 30 ml Phosphate buffer

- 1 mg/mL **fluorescamine** in acetonitrile (~1.76 mL for a full 96-well plate):

  Prepare enough for one or two weeks (5mg + 5mL ACN).
  Weigh on tiny, folded foil

- 200 mg/mL **Protein Standard stock** (Sigma bovine serum albumin (BSA)):

  diluted to 10 mg/mL working stock with phosphate buffer

- 0.5 µg/mL **Uroporphyrin I (UroI) Fluorescent Standard** stock in 1 N HCl:

  diluted to 0.5 uM UroI with 1 N HCl

- 6 N Hydrochloric acid (HCl)

IMPORTANT NOTE
Due to the volatility of acetonitrile, the new pipet tip must be conditioned before pipetting any solutions containing acetonitrile. This requires pipetting the solution up and down while the tip remains in the solution three to five times before transferring any liquid. Once the tip has been conditioned, it can be used to transfer additional fluid

from the solution with minimal conditioning if necessary (i.e., pipet up and down only once between transfers). The only exception is when transferring the extracted supernatant to a clean tube as this will disturb the pellet.

Extraction

1) Mark 1.5 mL tubes at ~70 µL mark by placing tube in 0.5 mL tube rack and mark bottom of tube with a Sharpie.

2) Transfer approximately 50 µL of frozen ground coral tissue to a chilled microcentrifuge tube using a chilled stainless steel spatula. Keep tube chilled in liquid nitrogen (LN) before and after tissue transfer. Samples are kept frozen at -80°C until extracted.

3) At the time of extraction, thaw samples on ice and add 500 µL of extraction buffer (25% acetonitrile in 50 mM phosphate buffer, pH 8.0) to each tube and quickly vortex to mix. NOTE: Twenty-six (26) samples can be analyzed at one time on a single plate. It is recommended to extract the samples in two separate batches. Keep one batch frozen while extracting the first.

4) Disrupt coral tissue using a Mini-Beadbeater-8 kept chilled at 4°C in a walk-in refrigerator. The tissue is homogenized without adding beads for two cycles of 1 min each at maximum speed with a one minute interval between each cycle. **NOTE**: Only eight (8) tubes will fit at a time in this bead-beater. Keep all the tubes in the walk-in while bead-beating.

5) Incubate the tissue homogenate at room temperature for 15 minutes, vortexing every 5 min to increase the interaction between coral tissue and extraction buffer. NOTE: During this incubation, proceed to extract the next batch of samples.

6) After incubating, centrifuge coral tissue homogenates at maximum speed (20,817 *x g*) for 15 min and remove the supernatant to a new amber microcentrifuge tube.

Protein Assay

1) Prepare a five-point protein standard from a 200 mg/ml Protein Standard stock (Sigma bovine serum albumin (BSA)). First make a 10 mg/ml BSA working stock by diluting the BSA Protein Standard 1:20 in phosphate buffer. **NOTE**: This can be started as the last batch of samples is spinning down.

2) Dilute the working stock of BSA to 800 µg/ml in extraction buffer and make two-fold serial dilutions by diluting 1:1 in extraction buffer to a final 50 µg/ml BSA standard. Extraction buffer without BSA is included as a blank.

3) Add 415 µL phosphate buffer to each of the tubes to be used in step #5 below.

4) Pre-read the 96-well Nunc® optical bottom black plate that will be used for the protein assay using the Bio-Tek® Synergy™ HT microplate reader fitted with 400 ± 15 nm excitation and 460 ± 20 nm emission filters. Set optics for bottom position at a sensitivity of 50.

5) In a new microcentrifuge tube, add 80 µL of coral tissue extract or BSA standard to 415 µL of phosphate buffer and vortex to mix.

6) Add 55 µL of **1 mg/mL fluorescamine** in acetonitrile and quickly vortex to mix.

7) Aliquot 150 µL of the mixture in triplicate to the pre-read 96-well Nunc® optical bottom black plate using suggested template. Cover wells once samples have been pipetted.

8) Within approximately 15-20 minutes after the last sample is mixed and all samples plated, measure protein fluorescence using the Bio-Tek® Synergy™ HT microplate reader with the same setting as above.

9) Generate a standard curve and calculate sample total soluble protein concentrations using the associated Bio-Tek® KC4 microplate data analysis software.

10) Using the determined concentrations, adjust samples to 10 µg/150 µL(66.7 µg/mL) total soluble protein by diluting an aliquot of the original extraction with extraction buffer in a new amber microcentrifuge tube. If a sample is not concentrated enough, use as much protein as possible based on the sample-well volume (~350 µL max).

Porphyrin Assay

1) Prepare a five-point set of standards from a 0.5 µg/mL **Uroporphyrin I (UroI) Fluorescent Standard stock** in 1 N HCl. UroI serves as a proxy for measuring total porphyrin concentration in the extracted coral tissue. First make a 0.5 µM UroI working stock by diluting the 0.5 µg/mL stock with 1 N HCl.

2) Prepare an initial 1.0 pmol **UroI** standard by diluting the UroI working stock to a final concentration of 1.0 pmol/150 µL (6.67 pmol/mL) in extraction buffer containing 10 µg/150 µL BSA (final concentration; obtained from the 800 µg/mL BSA standard prepared earlier).

3) Subsequent two-fold serial dilutions are prepared by diluting the 1.0 pmol UroI standard 1:1 in extraction buffer containing 10 µg/150 µL BSA (final) down to a final 0.0625 pmol UroI standard. Extraction buffer containing only 10 µg/150 µL BSA is included as a blank.

4) Pre-read a new 96-well Nunc® optical bottom black plate that will be used for the porphyrin assay using the Bio-Tek® Synergy™ HT microplate reader fitted with 400 ± 15 nm excitation and 600 ± 20 nm emission filters. Set optics for bottom position at a sensitivity of 125.

5) Aliquot 150 µL of diluted extract or UroI standard in triplicate to the new 96-well Nunc® optical bottom black plate using suggested template. Cover wells once samples have been pipetted.

6) Using a multichannel pipettor, add 30 µL of 6 N HCl to each well.

7) Using the Bio-Tek® microplate reader, immediately shake the plate two times for 20 s each at low intensity and incubate at room temperature covered in the dark for 10 min.

8) Measure porphyrin fluorescence using the Bio-Tek® microplate reader with the same settings as above (not those used for the protein assay).

9) Generate a standard curve and calculate sample porphyrin concentrations using the associated Bio-Tek® KC4 microplate data analysis software.

10) Express sample porphyrin content as fmol porphyrin per µg protein.

Additional Notes and Formulations:

Suggested plate template

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	BL	BL	S7	S7	S7	S14	S14	S14	STD4	STD4	STD4
B	S1	S1	S1	S8	S8	S8	S15	S15	S15	S21	S21	S21
C	S2	S2	S2	S9	S9	S9	STD3	STD3	STD3	S22	S22	S22
D	S3	S3	S3	S10	S10	S10	S16	S16	S16	S23	S23	S23
E	S4	S4	S4	STD2	STD2	STD2	S17	S17	S17	S24	S24	S24
F	S5	S5	S5	S11	S11	S11	S18	S18	S18	S25	S25	S25
G	STD1	STD1	STD1	S12	S12	S12	S19	S19	S19	S26	S26	S26
H	S6	S6	S6	S13	S13	S13	S20	S20	S20	STD5	STD5	STD5

Protein Standards

- 800, 400, 200, 100, 50 µg/mL **BSA** and blank. Load on plate lowest to highest concentration.
- **Blank** is extraction buffer without BSA
- **10 mg/mL BSA working stock:**

  dilute BSA standard 1:20 = 5 µL BSA + 95 µL phosphate buffer
- For 1.0 mL of **800 µg/mL BSA**:

  920 µL extraction buffer
  80 µL of 10 mg/mL BSA stock
- **Serially dilute** 200 µL of 800 µg/mL BSA into 200 µL extraction buffer

Porphyrin Standards

- 1.0, 0.5, 0.25, 0.125, 0.0625 pmol **UroI** and blank. Load lowest to highest concentration.
- **Blank** is porphyrin standard diluent without UroI

- For 3.6 mL of **porphyrin standard diluent**:

  3.3 mL extraction buffer (3*1000 µL + 300 µL)
  300 µL of 800 µg/mL BSA standard

- For 1.2 mL of **1.0 pmol UroI** in 10 µg/150 µL BSA:

  1084 µL extraction buffer
  100 µL of 800 µg/mL BSA standard (prepared earlier)
  16 µL of 0.5 µM UroI stock

- **Serially dilute** 600 µL of standard into 600 µL of standard diluent

Chemical List

- Acetonitrile (#270717; Sigma, St. Louis, MO)
- BSA Protein Standard (#P5369; Sigma, St. Louis, MO)
- Fluorescamine (#F2332; Life Technologies, Grand Island, NY)
- Hydrochloric acid (#258148; Sigma, St. Louis, MO)
- Sodium phosphate dibasic, anhydrous (#S9763; Sigma, St. Louis, MO)
- Sodium phosphate monobasic, monohydrate (#S9638; Sigma, St. Louis, MO)
- Uroporphyrin I (UroI) Fluorescent Standard (#UFS-1; Frontier Scientific, Logan, UT)

APPENDIX II.

Assessing the Utility of AFLP for Determining Mutational Changes in Coral

Cheryl M. Woodley[1], Darlene L. Middleton[2] and Lisa A. May[2]

[1]NOAA CCEHBR Hollings Marine Laboratory, 331 Ft. Johnson Rd., Charleston, SC 29412, USA
[2] JHT Inc. Contractor at National Oceanic & Atmospheric Administration, NOS CCEHBR Hollings Marine Laboratory, 331 Ft. Johnson Rd., Charleston, SC 29412, USA

Introduction

Degradation of the world's coral reefs is one of the most vivid examples of the effects of global environmental damage of marine ecosystems. The major stressors responsible for coral reef decline have been attributed to coastal urban and industrial development, agricultural activity, sedimentation, overharvesting, marine pollution, disease and climate change (Walker & Ormond 1982; Bryant *et al*. 1998; Risk 1999; Turgeon *et al*. 2002; Bellwood *et al*. 2004). Reef species experiencing persistent environmental disturbances (e.g., coastal development and land-based pollution) may exhibit acute mortality leading to a seemingly rapid loss of coral-reef diversity and abundance, but may also display non-acute, sub-lethal effects. These effects are often present as increased incidence of disease, altered growth and regeneration rates, reduced reproductive effort and reduced recruitment, which can ultimately result in a cascading effect of ecosystem deterioration (Richmond 1993; Hoegh-Guldberg 1999; Nystrom *et al*., 2000; Knowlton 2001; Porter & Tougas 2001; CRMP 2001; Patterson *et al.* 2002).

The ecological consequences to coral and coral reefs of exposures to increasing amounts and types of pollutants are difficult or impossible to assess in short-term (less than five years) studies. One of the most informative endpoints to measure

the risk of these exposures is reproductive fitness, though this too is logistically challenging, time-consuming and expensive. However, indirect measures of reproductive fitness can be made through carefully selected proxies that are well supported and accepted in the research community, and which reflect specific aspects of reproductive physiological condition. Markers that reflect conditions of genomic integrity provide a group of such proxies (Moore *et al*. 2004; Ricketts *et al*. 2004; Jha 2008)

Genotoxic compounds can act directly through the accumulation of deleterious mutations or indirectly by affecting the organism's physiology or environment, but in either instance they often affect survival and/or fecundity (De Wolf *et al*. 2004). Studying the direct effects of contaminants on DNA structure and function are important because DNA is the foundation of reproduction and inheritance, and changes in its structure or function often lead to population level changes, i.e., affecting population structure or demographics (Theodorakis 2001). There

are numerous techniques for assessing structural or functional changes in DNA. These include markers such as those measuring DNA damage (e.g., DNA abasic sites, COMET assay), DNA adducts (e.g., 8-oxo-dG) or mutations (e.g., RAPDs, SNPs, RFLPs, SSCPs). Cells are equipped with DNA repair systems and can combat the effects of genotoxic compounds, thus information from a single evaluation of DNA integrity provides only a snapshot of DNA damage to that organism. These assays cannot determine the net effect of such exposures. To address this gap in information, an assay that could determine accumulation of mutations over time would be a valuable tool for determining the risk of pollutant exposures to coral health. The AFLP technique was selected for evaluation because large portions of the genome could be sampled and it does not require *a priori* DNA sequence information. Two desirable features of this assay are that it is applicable to any species and is relatively low cost (Amar *et al*. 2008). Furthermore, since it is based on amplification of genomic restriction fragments, the technique is robust and reliable due to the assay's stringent conditions (Vos *et al*. 1995).

Rationale

The AFLP assay is a DNA fingerprinting technique, which involves electrophoresis of DNA fragments from an organism or cell sample in a gel matrix, to generate a unique banding profile. Most current DNA fingerprinting techniques use PCR to generate the fragments (e.g., RAPDs, DGGE, AP-PCR). The major disadvantage of these fingerprinting techniques is that they are sensitive to DNA quality, reaction conditions and reaction temperature profiles (Vos *et al*. 1995). Because of these issues, several of these techniques have come under significant criticism (Atienzar & Jha 2006).

The AFLP technique was originally developed by Vos *et al*. (1995) for genotyping individuals and has also been used for population structure analyses (Amar *et al*. 2008), and mutation rate determinations (Kropf *et al*. 2009). This technique overcomes weaknesses in other DNA fingerprinting assays by combining the specificity of DNA restriction enzymes with amplification of fragments by PCR.

There are four key steps to the method: 1) extraction and restriction digestion of genomic DNA, 2) ligation of adapters to the ends of the restriction fragments, 3) selective amplification of the modified fragments, and 4) electrophoresis of the resulting products. The genomic DNA of samples is digested with two different restriction enzymes, one with a 4-base and the other with a 6-base recognition sequence. Pre-selective PCR primers with two-base overhangs are matched with adapters related to the restriction enzyme recognition sites to selectively reduce the number of DNA fragments. Amplification reactions are performed using primers with three-base overhangs and labeled such that only fragments containing the restrictions sites used initially, will be detected. PCR products are then analyzed by gel or capillary electrophoresis. Fragments are binned and then analyzed for fingerprint similarity with other samples.

The objective of the two-enzyme restriction cut is to generate DNA fragments of optimal size for amplification and of a size easily separated on polyacrylamide denaturing gels. This double cut

strategy also reduces the number of fragments that will amplify to only a subset of the restriction fragments, using PCR primers for the adapters. If needed, this approach provides a means to selectively label one strand of the PCR products to prevent mobility differences between double strands. In addition, it allows optimization of fragment numbers while maintaining stringent assay conditions, not available in other DNA fingerprinting techniques (Vos *et al*. 1995).

Strategy

The AFLP assay has been commercialized by several companies (e.g., Life Technologies, Licor, Beckman Coulter, Applied Biosystems®) into a kit format for various platforms (e.g., capillary electrophoresis, gel-based systems). The Applied Biosystem kits (Amplification Core Mix Module, Cat. # 402005 and AFLP Ligation and Preselective Amplification kit, Cat. # 402004) were used as the basis for this evaluation. It should be noted that coral tissues include symbiotic algae and a unique surface microbial community that can be sources of non-coral DNA which could confound results (Amar *et al*. 2008). To minimize these sources of possible artifacts, a strategy was developed that uses the standard AFLP protocol to identify candidate coral genomic fragments and validate them by DNA cloning and sequencing. Validated coral genomic fragments are used to design an assay for assessing accumulation of mutations in corals exposed to potentially genotoxic compounds. The accumulation of mutations is then assessed on coral-specific fragments from field samples collected using a repeated measures design (i.e., resampling the same individual over time). Direct sequencing or RFLP-type analysis is used to quantify mutations. Finally a practical assay needs to be amenable to high-throughput analysis.

Standard AFLP Protocol

DNA isolation - Samples of *Porites lobata* previously collected from reference and impacted field sites were used as source materials for this evaluation. DNA was isolated from frozen cryomilled samples using the Get *pure*DNA kit (Dojindo Molecular Technologies), with a minor modification of the addition of polyvinylpolypyrrolidone (PVPP) to bind polyphenolic compounds inherent in coral tissues (May & Woodley In Press; also http://cdhc.noaa.gov/_docs/Virtual%20Chemiluminescent%20DNA%20AP%20Site%20Assay_formatted_11-15-11.pdf). Briefly, ~50 mg of frozen cryomilled coral tissue was placed into a 1.5 ml Eppendorf tube containing 400 µL of room temperature lysis buffer from the kit and ~15 mg of PVPP. The remaining steps were conducted according to the manufacturer's instructions. The DNA yield was determined using the Quant-iT™ kit (Invitrogen, Life Technologies) and its integrity by agarose gel electrophoresis.

Genomic DNA restriction digests - High molecular weight DNA (~100 ng) was double-digested with 500 units of *Eco*RI (50,000 units/ml; New England Biolabs), and 100 units of *Mse*I (10,000 units/ml; New England Biolabs), overnight at 37°C. Complete digestion of the genomic DNA was assessed by agarose gel electrophoresis.

Adapter ligation – Using the Applied Biosystems® AFLP kit (Life Technologies, Grand Island, NY) components, *Eco*RI and *Mse*I adaptors were ligated onto the fragmented DNA by

incubating overnight at room temperature with T4 DNA ligase. The adapters consist of a core sequence, an enzyme specific sequence, and a selective extension with usually three selective nucleotides (Vos *et al*. 1995).

Preselective PCR amplification – The first amplification used primers corresponding to each end of the modified restriction fragments plus one selective nucleotide to provide sufficient material for subsequent selective PCR. The template of genomic DNA fragments with modified adaptors was PCR amplified using pre-selective primers from the kit with the following conditions: initial denaturation at 94°C for 2 min, twenty cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 2 min, followed by a final extension of 60°C for 30 min and a hold at 4°C.

Selective PCR amplification - In a second amplification reaction, primers with selective nucleotides included at the 3' ends were used to amplify a subset of restriction sites, since only the restriction fragments having these specific nucleotides flanking the restriction site will match and be amplified. Eight pairs of selective primers (kit components) were evaluated using the test DNA for amplification to determine which pair provided optimal banding profiles. Reaction conditions for these tests were conducted according to the manufacturer's instructions, and included a touchdown annealing step from 66°C (-1 °C/cycle) for ten cycles followed by twenty cycles with a 56°C annealing temperature. Two primer pairs selected for further analysis were *Eco*RI-ACA/*Mse*I-CTA and *Eco*RI-ACA/*Mse*I-CAG.

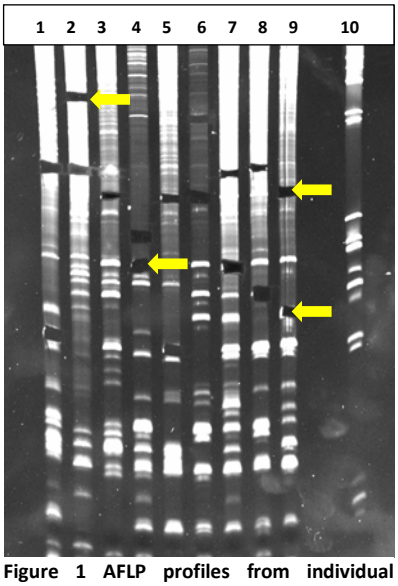


Figure 1 AFLP profiles from individual *Porites lobata* samples (lanes 1-9). Arrows indicate examples of the location where DNA fragments were cut from a 6% denaturing polyacrylamide gel for cloning. Lane 10 is a control.

Cloning of AFLP fragments-PCR products from selectively amplified AFLP DNA were subjected to denaturing polyacrylamide electrophoresis (6% polyacrylamide, 7 M urea) and bands were excised from each DNA sample, targeting those estimated to be ~1 kb (e.g., Fig. 1). Each DNA fragment was eluted from the excised gel in sterile deionized water at 4°C overnight and cloned into the pCR2.1 vector using a TOPO-TA cloning kit (Invitrogen).

Clones were evaluated for insert size by PCR amplification using primers from the multiple cloning sites of the vector and electrophoresis on a 0.8% agarose gel. All of the resulting inserts from the cloned fragments however were smaller than 500 bp. The fact that these fragments were smaller than anticipated is likely due to the denaturing gel electrophoresis conditions and also the standard AFLP protocol was designed for targets generally 1 kb or less. These small fragments were deemed unsuitable for determining mutation accumulation. It was concluded that for the intended application, the procedure required modification to obtain larger fragments.

Modified AFLP Protocol

Modification of AFLP protocol to increase fragment size – Three options were considered to increase the fragment sizes: 1) change the enzyme combinations in the initial genomic digest, 2) conduct blunt-end digestions, or 3) digest the genomic DNA with *Eco*RI alone. Option three was selected for further evaluation because it was the most efficient while continuing to provide a means of selective amplification.

The AFLP protocol was followed essentially as described above except only one enzyme was used in the restriction digest, *Eco*RI. Accordingly, twice the amount of *Eco*RI adaptors were used and the *Mse*I adaptors were omitted from the ligation reaction. The pre-amplification products then were amplified with the *Eco*RI selective primer *Eco*RI-ACA only. Products were separated in a 6% denaturing polyacrylamide gel and bands cut from the gel and eluted as described above. Re-amplification of these bands again yielded fragments <600 bp.

With the goal of producing larger fragments, two new selective primer sets (EcoRI-ACA/EcoRI-AAG and EcoRI-AAG/EcoRI-AAC) were evaluated. Amplification products were separated on a 6% denaturing polyacrylamide gel that was run for 20 h at 400V. The extended electrophoresis time and lower voltage as compared to previous runs were used to separate the largest fragments from the amplification products. A subset of bands was cut from this gel and eluted. The eluted DNA was re-amplified using the same primer sets and evaluated for fragment size and number on a 1% agarose gel (Fig. 2). These conditions yielded many large fragments of approximately 1 kb in length. These fragments were cloned and 96 clones were sequenced.

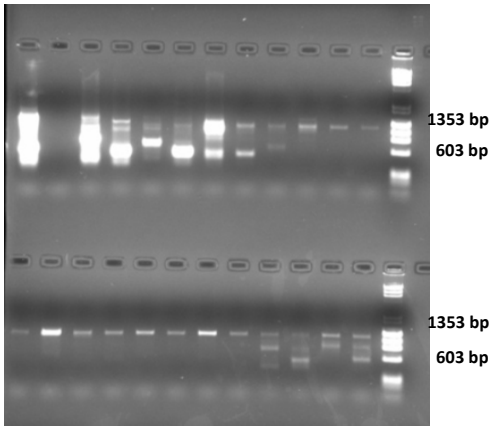


Figure 2 PCR products of excised bands separated in 0.8% agarose gel showing fragments >1 kb (lanes 1-12 top & bottom). Lane 13 top & bottom HaeIII digested ØX174 MW marker.

Following a Basic Local Alignment Search Tool (BLAST) (Altschul *et al*. 1990) analysis of the cloned DNA, seven target coral sequences were identified. Oligonucleotide primers were designed to target each of the seven putative coral sequences as shown in Table I. Primer pairs were tested against 50 field samples to determine whether individual fragments could be amplified from multiple colonies of a given species and thus meet a critical criterion. Test samples included *Porites lobata* from Hawaii: Maunalua Bay (6 subsites, 23 colonies), Ordnance Reef (2 subsites, 7 colonies), West Maui (2 subsites, 17 colonies), and La Perouse (1 subsite, 3 colonies). Additionally, the primers were tested on a cultured coral, *Pocillopora damicornis* (5 colonies) from the CCEHBR

Coral Husbandry Facility, Charleston, SC.

Results

The modifications made to the standard AFLP protocol did yield larger sized AFLP fragments (Fig. 2). However, when AFLP fragments were cloned and sequenced, the BLAST results indicated that many of the clones were of bacterial origin (data not shown). This confirmed our suspicions that the standard AFLP method was not coral-specific in the banding profiles it generated and thus could not be used to determine mutational changes for coral. Thus, our strategy to isolate coral-specific genomic fragments was necessary to be able to assess mutation frequency in coral, however to now use coral-specific genomic fragments would require designing a new assay platform.

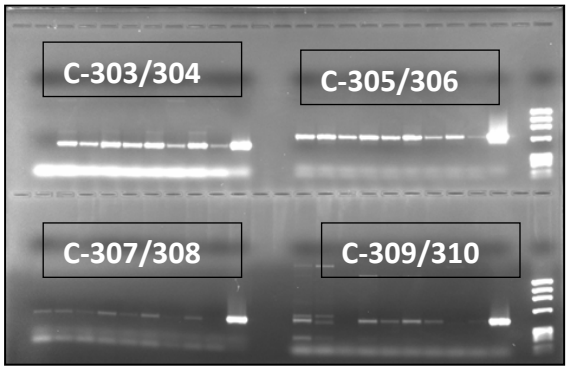


Figure 3 Screening field samples of *Porites lobata* for fragment amplification across individual coral colonies with primer sets designed from cloned *P. lobata Eco*RI restriction fragments. Lane 25, top & bottom MW markers. Gel 0.8% agarose.

In an effort to design a screening assay to detect increased mutations in corals exposed to potentially genotoxic pollutants, seven coral-specific genomic sequences >1kb that had been isolated from AFLP polyacrylamide gels, cloned and verified by DNA sequencing (gel not shown) were used to design PCR primers for testing individual specimens. PCR products of the correct target size indicated that the corresponding restriction sites (at the end of each fragment) were intact across individuals. All primer pairs except C315F/C316R successfully amplified the target coral sequences, but required predigestion of the genomic DNA with *Eco*RI for a successful amplification (Fig. 3 example of agrose gel screening). Oligonucleotide primers, C315F/C316R, failed to amplify any *P. lobata* samples, possibly due to inaccuracies in the sequence data, or *Eco*RI star activity during the template restriction digest. In some instances, only a few *P. lobata* samples failed to yield PCR products with certain primer pairs. The oligonucleotide primer combination that successfully amplified targets from the highest number of samples was C303/C304 (amplified 76.1 % of samples). Primer pair C313/C314 had the lowest rate of success with only 16.4 % of coral samples amplified. All other primer pairs amplified between 58.2- 69.1 % of samples.

Conclusions

Two variations of the AFLP technique were used in an attempt to develop an assay to screen coral for mutation accumulations. The first used the standard AFLP protocol. Results yielded small fragments <500 bp and cloning and sequencing efforts of a subset of DNA fragments showed the presence of DNA from other organisms resident in the holobiont, making this approach unsuitable for directed monitoring of coral mutational events.

The second approach was a modification of the standard AFLP protocol that involved carrying out the procedure using only one restriction enzyme, *Eco*RI with a six-base recognition sequence. This modification did yield fragments approximately 1-1.3 kb. However, subsequent validation procedures yielded only seven coral-like DNA fragments for further analysis. Eight primer combinations of 64 possible combinations were tested. No primer combination tested amplified all samples.

From these experiments, we concluded that the AFLP assay as executed to this point is not suitable for determining mutational changes in coral. Though the modifications implemented did provide a means of tracking coral-specific restriction fragments, in most cases the sequences obtained did not correspond to a particular gene fragment. This eliminated the possibility of determining possible functional consequences of a mutation. Further, the amount of genome coverage was small, so even if multiple fragments were used in the screening assay it is unlikely there would be sufficient sensitivity to detect even moderate-levels of mutational events over a one to three month timeframe (repeated measures sampling frequency) without further modifications.

Abbreviations:

AP-PCR-Arbitrarily primed polymerase chain reaction
DGGE-Denaturing gradient gel electrophoresis
RAPDs–Random amplification of polymorphic DNA
RFLP-Restriction fragment length polymorphism
SNP-Single-nucleotide polymorphism
SSCP-Single-strand conformation polymorphism
8-oxo-dG - 8-oxo-7,8-dihydro-2'-deoxyguanosine

Table 1. PCR Primers for Coral Genomic Fragments

Primer Designation	Source Species	Source Site	Source Colony	Primer Sequence (5′-3′)	Pair with Primer
C303F	*Porites lobata*	Ordnance Reef, Oahu, HI	1-3	AAA GAC GCT CAG TGT TGG GT	C304R
C304R	*Porites lobata*	Ordnance Reef, Oahu, HI	1-3	AAT TGC TGT GCG GCA TTG AA	C303F
C305F	*Porites lobata*	Ordnance Reef, Oahu, HI	1-2	GCC TGA ACA ATG CAA AGC CA	C306R
C306R	*Porites lobata*	Ordnance Reef, Oahu, HI	1-2	GTT GAG CCG GCG ACT AGT AA	C305F
C307F	*Porites lobata*	Maunalua Bay, Oahu, HI	K5	TTC CAT CAT GGT CGT GCA GT	C308R
C308R	*Porites lobata*	Maunalua Bay, Oahu, HI	K5	TAG GTG GGG AAT CAA ACG GC	C307F
C309F	*Porites lobata*	La Perouse, Maui, HI	3-3	GCT GGC TTA CAG GGT AGC AG	C310R
C310R	*Porites lobata*	La Perouse, Maui, HI	3-3	TGC CTA ATG TGG CAC CAA GT	C309F
C311F	*Porites lobata*	La Perouse, Maui, HI	3-3	TAA GGT CTC CCC GAC CGA TT	C312R
C312R	*Porites lobata*	La Perouse, Maui, HI	3-3	ACC AAA AAG AAT CGC CGT GC	C311F
C313F	*Porites lobata*	La Perouse, Maui, HI	3-3	CGT CCG AGA AGT ACG TTC CA	C314R
C314R	*Porites lobata*	La Perouse, Maui, HI	3-3	CTT CTG AGG CTG GTA GGC TG	C313F
C315F	*Porites lobata*	Maunalua Bay, Oahu, HI	K5	TTG CTA TCC CCC AAA CCA CC	C316R
C316R	*Porites lobata*	Maunalua Bay, Oahu, HI	K5	TCT CTT TTT GGG GCG GGA AA	C315F

References

Amar, K., Douek, J., Rabinowitz, C., & Rinkevich, B. 2008. Employing of the Amplified Fragment Length Polymorphism (AFLP) methodology as an efficient population genetic tool for symbiotic cnidarians. Mar. Biotechnol. 10, 350-357.

Atienzar, F.A. & Jha, A.N. 2006. The random amplified polymorphic DNA (RAPD) assay and related techniques applied to genotoxicity and carcinogenesis studies: A critical review. Rev. Mut. Res. 613, 76-102.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., & Lipman, D.J. 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403-410.

Bellwood, D.R., Hughes, T.P., Folke, C. & Nystrom, M. 2004. Confronting the coral reef crisis. Nature. 429, 827-833.

Bryant, D., Burke, L., McManus, J. & Spalding, M. 1998. Reefs at Risk: A map-based indicator of threats to the world's coral reefs. Washington, DC: World Resources Institute. Pp. 56.

Coral/Hardbottom Monitoring Project (CRMP) Executive Summary 1996-2000, Steering Committee Report, August 1, 2001.

De Wolf, H., Blust, R., & Backeljau, T. 2004. The use of RAPD in ecotoxicology. Mutat. Res. 566, 249-262.

Flot, J.-F. & S. Tillier. 2007. The mitochondrial genome of *Pocillopora* (Cnidaria: Scleractinia) contains two variable regions: The putative D-loop and a novel ORF of unknown function. Gene 401:80-87.

Hoegh-Guldberg, O. 1999. Climate change, coral bleaching and the future of the world's coral Reefs. Mar. Freshwater Res. 50, 839-66.

Jha, A.N. 2008. Ecotoxicological applications and significance of the comet assay. Mutagenesis. 23, 207-221.

Knowlton, N. 2001. The future of coral reefs. Proc. Nat. Acad. Sci. U.S.A. 98, 5419-5425.

Kropf, M., Comes, H.P., & Kadereit, J.W. 2009. An AFLP clock for the absolute dating of shallow-time evolutionary history based on the intraspecific divergence of southwestern European alpine plant species. Mol. Ecol. 18, 697-708.

May, L.A. & Woodley, C.M. (in press) Chemiluminescent DNA AP site assay. In: *Diseases of Coral*. Wiley-Blackwell, Boston.

Moore, M.N., Depledge, M.N., Readman, J.W. & Leonard, D.R.P. 2004. An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. Mutat. Res. 552, 247-268.

Nystrom, M., Folke, C. & Moberg, F. 2000. Coral reef disturbance and resilience in a human-dominated environment. TREE 15, 413-417.

Patterson, K.L., Porter, J.W., Ritchie, K.B., Polson, S.W., Mueller, E., Peters, E.C., Santavy, D.L., & Smith, G.W. 2002. The etiology of white pox, a lethal disease of the Caribbean elkhorn coral, *Acropora palmata*. Proc. Natl. Acad. Sci. U.S.A. 99, 8725-30.

Porter, J.W. & Tougas, J.I. 2001. Reef Ecosystems: Threats to their biodiversity. In: *Encyclopedia of Biodiversity*. (ed. S. Levin), Academic Press. 5, 73-95.

Richmond, R.H. 1993. Coral reefs: Present problems and future concerns resulting from anthropogenic disturbance. Am. Zool. 33, 524-536.

Ricketts, H.J., Morgan, A.J., Spurgeon, D.J., & Kille, P. 2004. Measurement of annetocin gene expression: a new reproductive biomarker in earthworm ecotoxicology. Ecotox. Environ. Saf. 57, 4-10.

Risk, M.J. 1999. Paradise lost: how marine science failed the world's coral reefs. Mar. Freshwater Res. 50, 831-37.

Theodorakis, C.W. 2001. Integration of genotoxic and population genetic endpoints in biomonitoring and risk assessment. Ecotoxicol. 10, 245-256.

Turgeon, D.D., R.G. Asch, B.D. Causey, R.E. Dodge, W. Jaap, K. Banks, J. Delaney, B. D. Keller, R. Speiler, C.A. Matos, J.R. Garcia, E. Diaz, D. Catanzaro, C.S. Rogers, Z. Hillis-Starr, R. Nemeth, M. Taylor, G.P. Schmahl, M.W. Miller, D.A. Gulko, J.E. Maragos, A.M. Friedlander, C.L. Hunter, R.S. Brainard, P. Craig, R.H. Richmond, G. Davis, J. Starmer, M. Trianni, P. Houk, C.E. Birkeland, A. Edward, Y. Golbuu, J. Gutierrez, N. Idechong, G. Paulay, A. Tafileichig, and N. Vander Velde. 2002. The State of Coral Reef Ecosystems of the United States and Pacific Freely Associated States: 2002. National Oceanic and Atmospheric Administration, National Ocean Service, National Centers for Coastal Ocean Science, Silver Spring, MD. 265pp.

Vos, P., Hogers, R., Bleeker, M., Reijans, M. van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. & Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23, 4407-4414.

Walker, D.I. & Ormond, R.F.G. 1982. Coral death from sewage and phosphate pollution at Aqaba, Red Sea. Mar. Poll. Bull. 13, 21-25.

APPENDIX III.

Surveyor® Mutation Analysis of Coral Mitochondrial DNA
Lisa A. May

For an initial screen, select two tissue replicates from a 96 h exposure for these treatments: Carrier, Lowest Dose, Medium Dose, and Highest Dose (8 total samples). Tissue from the dosing experiment should be homogenized in liquid nitrogen (using mortar and pestle, or cryomill), then archived at -80 °C until use.

Coral Genomic DNA isolated with Qiagen Genomic-tip 20/G kit (recommended by Hunter, et al. 2010 for high quality nucleic acid)

1. Add to a sterile 15 mL polypropylene tube: 2.0 mL G2 Buffer (from kit, room temperature), 40 µL 10 mg/mL RNase A, and 100 µL proteinase K. Vortex to mix.
2. Place one medium scoop (~ 60-80 mg, using a pre-chilled stainless steel spatula) homogenized coral tissue in tube and vortex 5-10 s to mix.
3. Place tube at 50 °C for 2 h. Occasionally invert tube to mix during incubation. (Final lysate should be clear).
4. While tissue is incubating, set up 20/G tips with yellow suspension frames (from kit) over 15 mL polypropylene tubes and equilibrate each filter with 2.0 mL Buffer QBT. Allow tubes to empty by gravity flow (~5 min).
5. Warm QF Buffer to 50 °C.
6. Place clear lysate (do not disturb sediment at bottom of lysis tube) in a clean 1.5 mL tube and centrifuge at room temperature at 6,000 X g for 2-3 min to remove any particulate matter. Transfer supernatant to 20/G tip and allow to flow through column (gravity, 4-10 drops/min).
7. Wash each 20/G tip 3 times with 1.0 mL Buffer QC (gravity flow). Discard flow-through liquid.
8. Place 20/G tip with DNA into a clean 15 mL tube.
9. Elute DNA with 2 X 0.8 mL warmed Buffer QF (gravity flow).
10. Transfer eluent to two 1.5 mL tubes (TUBE 1 and TUBE 2, 0.8 mL each) and add 0.56 mL room temperature isopropanol to each.
11. Invert to mix and centrifuge immediately at 14,000 x g for 15 min at 4 °C.
12. Carefully aspirate supernatant and discard.
13. Wash DNA pellet (may not be visible) with 500 µL cold 70% ethanol.
14. Vortex briefly and centrifuge at 14,000 x g for 10 min at 4 °C.
15. Carefully aspirate supernatant and discard.
16. Air dry pellet for 15-20 min. Ensure that alcohol has dissipated.
17. Resuspend DNA in each tube with 50 µL TE (gently mixing by flicking tube with finger).

18. Dissolve DNA at 55 °C for 1-2 h. Following incubation, spin tubes quickly in microfuge, then pool DNA from replicate samples.
19. Verify integrity of genomic DNA by running 10 µL of each sample plus 2 µL tracking dye on 0.5% TAE-agarose gel containing 0.25 µg/mL ethidium bromide at 60 V for 2 h. Use Lambda-HindIII digest size standards (0.5 µg).
20. Image gel for records.

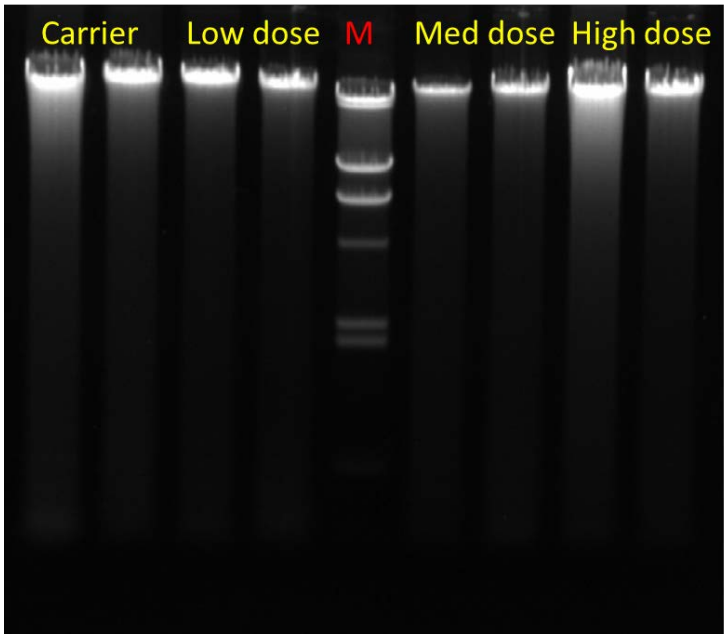


Figure 1. ***Pocillopora damicornis*** **genomic DNA isolation from coral tissue homogenate after 96 h exposure to munition compound RDX.** Ten microliters of each DNA sample (from experimental tissue replicates) were electrophoresed on a 0.5% agarose gel with Lambda-*Hin*d III DNA size markers (Lane M).

DNA Quantification using the Quant-iT™ High Sensitivity DNA Quantification kit and Qubit® fluorometer (Life Technologies, Grand Island, NY)

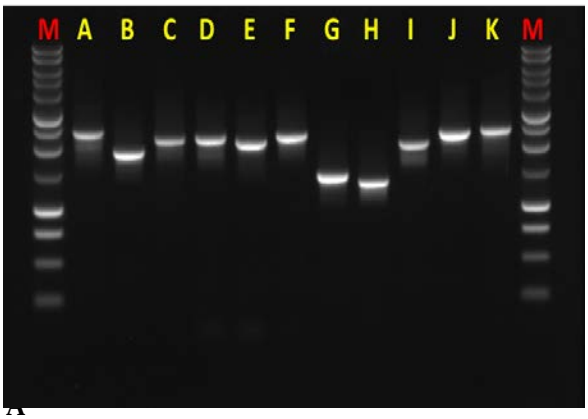
1. Prepare a 1:200 dilution of Quant-iT™ HS DNA dye in buffer solution.
2. Place 198 µL Quant-iT™ buffer solution (with HS DNA dye) into a 0.5 mL clear (PCR) tube.
3. Add 2 µL of well-mixed genomic DNA sample and vortex to mix. Incubate 2 min in dark.
4. Calibrate instrument with standards as per manufacturer's protocol, then read sample.
5. Multiply Qubit® raw values by 100 (dilution factor) for calculation of stock DNA sample. Recovery should be approximately 15-50 ng/µL.
6. Store 150-200 ng aliquots of each genomic DNA sample at -20°C.

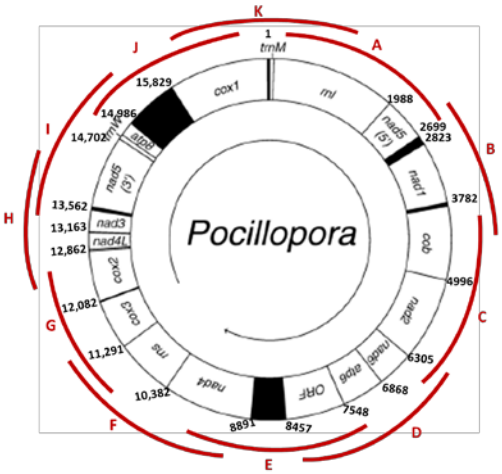
Surveyor® Mutation Detection Analysis for mitochondrial DNA (Bannwarth, et al. 2006)

1. Prepare two Surveyor® Control C and one Surveyor® Control G reaction tubes according to the manufacturer's protocol, and amplify as directed. This requires different cycling parameters from the coral samples and may be done ahead of time to use for several experiments. Products (632 bp) should be checked on an agarose gel (1.2%) and quantified with the Qubit® fluorometer, as with the coral genomic DNA PCR products.
2. Dilute coral DNA to ~0.38-0.50 ng/µL final concentration using sterile water.
3. Set up polymerase chain reaction using a primer set to amplify 1.5-2.5 Kb regions of the mtDNA genome. (See Appendix Tables for sequence information and expected product size of each primer set, A-K.)
4. Place 16 µL coral template samples in a thin-walled tube (6-8 ng). Note: prepare 2 tubes of untreated (carrier) control sample for each primer set to be analyzed. You will need at least one tube of carrier control for every 8 dosing samples to be analyzed, plus one tube for an uncut control.
5. Prepare a negative control for each primer set using 16 µL sterile water.
6. EXAMPLE for each of 11 primer sets: These reactions can be done over several days if necessary and stored at -20 °C until ready for the hybridization. (*110 total reaction tubes*).
    a. Carrier Control 1
    b. Carrier Control 1
    c. Carrier Control 2
    d. Low Dose 1
    e. Low Dose 2
    f. Medium Dose 1
    g. Medium Dose 2
    h. High Dose 1
    i. High Dose 2
    j. Negative Control
7. Reaction mixture/tube: Keep tubes on ice. Primer sets designed for *Pocillopora damicornis* and *Porites* sp. coral mitochondrial DNA are listed in Tables 1 and 2.
    a. 5 µL 10X ExTaq™ buffer
    b. 1 µL dNTPs (2.5 mM)
    c. 2 µL 10µM F primer (20 pmol)
    d. 2 µL 10µM R primer (20 pmol)
    e. 0.25 µL ExTaq™ polymerase
    f. 23.75 µL sterile MilliQ water
    g. Mix well and add 34 µL to each thin-walled tube containing template DNA.
8. Cycling parameters:
    a. 94 °C, 5 min
    b. 94 °C, 30 s
    c. 60 °C, 30 s

d. 72 °C, 3 min
e. repeat b-d 34 X
f. 72 °C, 10 min
g. 4 °C, HOLD

9. Evaluate 5 µL PCR products on a 1.2% TAE-agarose gel containing ethidium bromide. Ensure NO secondary amplification products are present for each sample, including primer dimers. Quantify PCR products using 2 µL of each sample in the Qubit® HS dsDNA assay according to the manufacturer's protocol. Target DNA concentration is 25-80 ng/µL.





Amplification fragments from *Pocillopora damicornis* mitochondrial DNA. Agarose gel lane M= 1 Kb DNA size ladder (Promega), A-K= *P. damicornis* PCR products from respective primer sets.

10. Heteroduplex formation <u>for each primer set</u>:
    a. Control 1, 300 ng Surveyor® Nuclease Control C
    b. Control 2, 150 ng Surveyor® Nuclease Control C and 150 ng Plasmid Control G
    c. Controls 3 and 4, 300 ng untreated DNA in a tube (150 ng Carrier 1 + 150 ng Carrier 2) (<u>prepare 2 tubes of this control</u>).
    d. Treatment samples, 150 ng of untreated (Carrier) and 150 ng treated DNA sample in a tube. Do this for all treatment samples
    e. EXAMPLE (one primer set):
        i. Plasmid Control C + Plasmid Control C
        ii. Plasmid Control C + Plasmid Control G
        iii. Carrier 1 + Carrier 2 (Uncut Control)
        iv. Carrier 1 + Carrier 2 (Digested Carrier Control)
        v. Carrier 1 + Low 1
        vi. Carrier 1 + Low 2
        vii. Carrier 1 + Medium 1
        viii. Carrier 1 + Medium 2

ix. Carrier 1 + High 1
x. Carrier 1 + High 2

f. Bring volume of all tubes to 12 µL with sterile water.
g. Place tubes in thermocycler, and hybridize:
i. 95 °C, 10 min
ii. 95-85 °C, -2.0 °C/s
iii. 85 °C, 1 min
iv. 85-75 °C, -0.3 °C/s
v. 75 °C, 1 min
vi. 75-65 °C, -0.3 °C/s
vii. 65 °C, 1 min
viii. 65-55 °C, -0.3 °C/s
ix. 55 °C, 1 min
x. 55-45 °C, -0.3 °C/s
xi. 45 °C, 1 min
xii. 45-35 °C, -0.3 °C/s
xiii. 35 °C, 1 min
xiv. 35-25 °C, -0.3 °C/s
xv. 25 °C, 1 min
xvi. 4 °C HOLD

11. Remove one tube of (Carrier 1 + Carrier 2, tube iii) and set on ice. This will be the Uncut Control (U).
12. Nuclease treatment should be optimized for the particular sample with respect to nuclease concentration (0.25-1.0 µL/reaction tube) and incubation time (20-60 min).

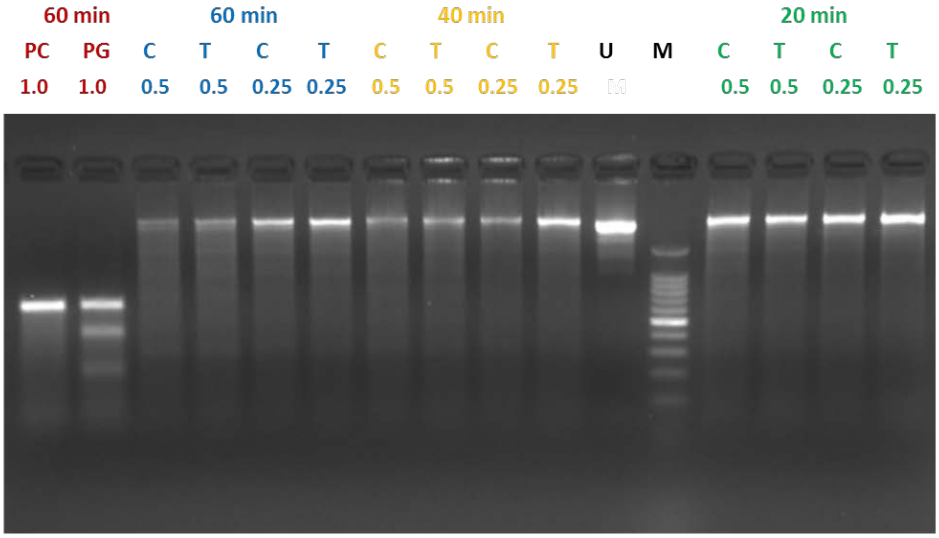


Figure 3. Results of nuclease concentration and incubation time test experiment using 0.25-1.0 µL enzyme and 20-60 min incubations on coral mtDNA amplification products. Lanes: PC=plasmid control, PG= plasmid mutation control, C=coral carrier control, T=treated coral sample, U= uncut coral sample, M=100 bp DNA size ladder. Coral samples treated with 0.5 µL nuclease for 20 min gave optimal results.

13. Coral samples worked optimally using these parameters: ($MgCl_2$, Enhancer and Nuclease is prepared as a cocktail, 2.7 µL/tube added to each PCR tube and pipetted up and down to mix)
    a. 12 µL hybridized DNA (300 ng)
    b. 1.2 µL 150 mM $MgCl_2$
    c. 1 µL Surveyor® Enhancer S
    d. 0.5 µL Nuclease S
14. Nuclease treatment for plasmid controls C and C/G
    a. 12 µL hybridized DNA (300 ng)
    b. 1.2 µL 150 mM $MgCl_2$
    c. 1 µL Surveyor® Enhancer S
    d. 1 µL Nuclease S
15. Heat Control C and Control C/G samples at 42 °C for 60 min and coral samples at 42 °C for 20 min. Begin the Control C and Control C/G reactions 40 min prior to beginning the coral samples.
16. Following incubation, add 1.5 µL STOP solution to each tube and pipet up and down to mix.
17. Add 3.5 µL 6X loading buffer to each tube, mix well, and evaluate all restriction products immediately on a 2.0 % TAE-agarose gel with ethidium bromide. Ensure that the wells

of the gel are deep enough to accommodate 20 µL of sample. Use an aliquot (300 ng) of hybridized, but undigested control sample (U) for reference (from step 11).

18. EXAMPLE for one primer set:

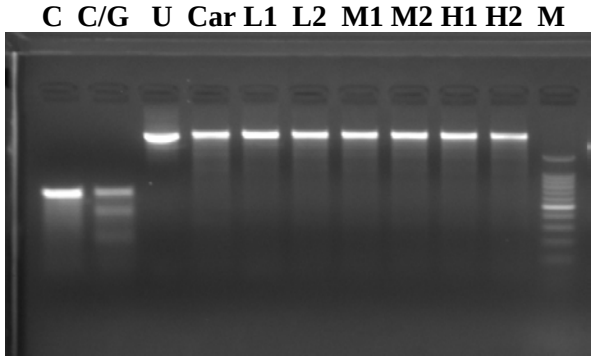


Figure 4. Results of Surveyor® Analysis on *Pocillopora damicornis* mtDNA exposed to 96 h RDX treatments (Primer set J). C=Plasmid control, C/G=Plasmid with single base mutation, U=Uncut coral control, Car=Carrier control, L1 and L2= Low treatment dose replicates, M1 and M2= Mid-range treatment dose replicates, H1 and H2=High treatment dose replicates, M=100 bp DNA size standards. No mutations were detected.

Table 1. *Pocillopora damicornis* oligonucleotide primers used to amplify 1.5-2.5 kb sections of the coral mitochondrial genome.

Primer Name	Fragment	Sequence (5'-3')	Length (bp)	C+G	Tm	Ta	Product Size	Reference
Pdam mtDNA rnlF	A	GTT AGT ACA AAT AGT CCG TCG CC	23	47.8%	55 °C	60 °C	2.47Kb	this work
Pdam mtDNA nad5(5')R	A	CCA TTG CAT CCG GTA ACC AAG TAT G	25	48.0%	58 °C	60 °C		this work
Pdam mtDNA nad5(5')F	B	GGA GAT CCT CAT ATT CCT CGA TTT ATG	27	40.7%	57 °C	60 °C	1.96Kb	Flot and Tillier 2007, revised
Pdam mtDNA cobR	B	GTC CTC AAG GCA AAA CAT AGC CC	23	52.2%	57 °C	60 °C		this work
Pdam mtDNA cobF	C	CTT CAT GCT AAC GGT GCT TCT TTG	24	45.8%	56 °C	60 °C	2.28Kb	this work
Pdam mtDNA nad6R	C	CGC TGA AAC AAC CAT TAC TCC TGA GCC	27	51.9%	61°C	60 °C		this work
Pdam mtDNA nad2F	D	GCA GGA ATT CCT CCT TTT GCT GGC	24	54.2%	59 °C	60 °C	2.31Kb	this work
Pdam mtDNA orfR	D	CAC ACA TGA GCC ATC ATC CCT TC	23	52.2%	57 °C	60 °C		this work
Pdam mtDNA atp6F	E	CGC TAT TAG AGG TGG CAG TTG C	22	54.5%	57 °C	60 °C	2.19Kb	this work
Pdam mtDNA nad4R	E	GGA GCC TCA ACA TGT GCT TGT GGC	24	58.3%	61 °C	60 °C		this work
Pdam mtDNA nad4F	F	CAT CAT AGG GAC AGC GAG GGA GAG	24	58.3%	59 °C	60 °C	2.35Kb	this work
Pdam mtDNA cox3R	F	GAG AAG GCT CAA CCA AAT GAT A	22	40.9%	51 °C	60 °C		Flot and Tillier 2007
Pdam mtDNA rnsF	G	GTT AGT ACA AAT AGT CCG TCG CC	23	47.8%	55 °C	60 °C	1.48Kb	Flot and Tillier 2007
Pdam mtDNA cox2R	G	GCC CTA AAG AAG GCA CTG C	19	57.9%	53 °C	60 °C		this work
Pdam mtDNA cox2F	H	GAA GGG GAT ACG TTA GGG TTT G	22	50.0%	55 °C	60 °C	1.38Kb	this work
Pdam mtDNA nad5(3')R	H	CCG CAT GAA TAA GAG ACC CTG CAC TC	26	53.8%	61 °C	60 °C		this work
Pdam mtDNA nad3F	I	CCA GAT CGA GAA AAG GTT TCT GC	23	47.8%	55 °C	60 °C	2.14Kb	this work
Pdam mtDNA int11R	I	GAA CTG AAA GAC CCG CAT CTC CC	23	56.5%	59 °C	60 °C		this work
Pdam mtDNA atp8F	J	GCC ACA GTT AGA GGT AGG TAC	21	52.4%	54 °C	60 °C	2.38Kb	this work
Pdam mtDNA cox1R	J	CGT CTT GGA AAT CCT GCT AAA CC	23	47.8%	55 °C	60 °C		Flot and Tillier 2007, revised
Pdam mtDNA cox1F	K	CAA GCA CAC TCC GGA GGT TCT G	22	59.1%	59 °C	60 °C	2.51Kb	this work
Pdam mtDNA rnlR	K	CGC TAC ATT ATC ACA GTC AGT G	22	45.5%	53 °C	60 °C		this work

Table 2. *Porites* sp. oligonucleotide primers used to amplify 1.5-2.5 kb sections of the coral mitochondrial genome.

Name	Fragment	Sequence (5'-3')	Length (bp)	C+G	Tm	Ta	Product Size	Reference
Porites mtDNA nad5(5')F	A	GGT GCT GGA ATT TTA ACT TCA AGC	24	41.7%	54°C		2.08 Kb	this work
Porites mtDNA cobR	A	GAT TCT CTT TGC GCA GTG GCA TAG G	25	52.0%	59°C			this work
Porites mtDNA nad1F	B	CGG TAT GAT CAG CTT ATG GCT C	22	50.0%	55°C		2.28 Kb	this work
Porites mtDNA nad2R	B	CAG ACC AGA TGA AAG TGC ACC	21	52.4%	54°C			this work
Porites mtDNA intF	C	GAT GGT GGA CAC GGA AAA GC	20	55.0%	54°C		2.34 Kb	this work
Porites mtDNA atp6R	C	CGA CAC CAT GAA GAT GAT CAT AG	23	43.5%	53°C			this work
Porites mtDNA nad6F	D	CTT GAG ATT TGG CAA CTC CTT GG	23	47.8%	55°C		2.49 Kb	this work
Porites mtDNA nad4R	D	CAA ACC CGT GCG CTA ACA TCA TG	23	52.2%	57°C			this work
Porites mtDNA nad4F	E	GCC TCC GAG TAT TTT GCT CCT C	22	54.5%	57°C		2.23 Kb	this work
Porites mtDNA cox3R	E	GGT CAA GCC ACA CCC AAT TCA AC	23	52.2%	57°C			this work
Porites mtDNA cox3F	F	GCG AAC TGT TTT ATG TCA TCC	21	42.9%	50°C		1.50 Kb	this work
Porites mtDNA nad5(3')R	F	GGA GCT TGT TCA AAA AGA GGA GAA G	25	44.0%	56°C			this work
Porites mtDNA nad3F	G	CTT TGG GTT TAC TCT ATG AGT G	22	40.9%	51°C		2.13 Kb	this work
Porites mtDNA cox1(5')R	G	CAT TGC ACC CAA AAT CGA GGA C	22	50.0%	55°C			this work
Porites mtDNA cox1(5')F	H	CTA CTA ACC ATA AAG ACA TTG GTA CG	26	38.5%	55°C		2.19 Kb	this work
Porites mtDNA coxintR	H	GAG CAC CCT TCT TCC CAC TAT GC	23	56.5%	59°C			this work
Porites mtDNA coxintF	I	CTA GGG TCA ATC AGT GGG AAA C	22	50.0%	55°C		2.17 Kb	this work
Porites mtDNA rnlR1	I	CTC GAC CTT CTC TTC ACC TAC	21	52.4%	54°C			this work
Porites mtDNA trnMF	J	GTA GAG AAG ACG AAT GGT GAG TC	23	47.8%	55°C		2.10 Kb	this work
Porites mtDNA rnlR2	J	GAA ACC AAG CTG TGT TAC CAC GC	23	52.2%	57°C			this work
Porites mtDNA rnlF	K	GCT TGG TAG TAG AAC AGA CTG	21	47.6%	52°C		2.14 Kb	this work
Porites mtDNA nad5(5')R	K	CCA ACT GTG CAG ACT TTC CAA CC	23	52.2%	57°C			this work

References and further reading:

Bannwarth. S, V. Procaccio, and V. Paquis-Flucklinger. 2006. Rapid identification of unknown heteroplasmic mutations across the entire human mitochondrial genome with mismatch-specific Surveyor® Nuclease. Nature Protocols. **1**:2037-2047.

Chen, C., C.-Y. Chiou, C.-F. Dai, and C. A. Chen. 2008. Unique mitogenomic features in the scleractinian family Pocilloporidae (Scleractinia:Astrocoeniina). Mar. Biotechnol. **10**:538-553.

Flot, J.-F. and S. Tillier. 2007. The mitochondrial genome of *Pocillopora* (Cnidaria: Scleractinia) contains two variable regions: The putative D-loop and a novel ORF of unknown function. Gene **401**:80-87.

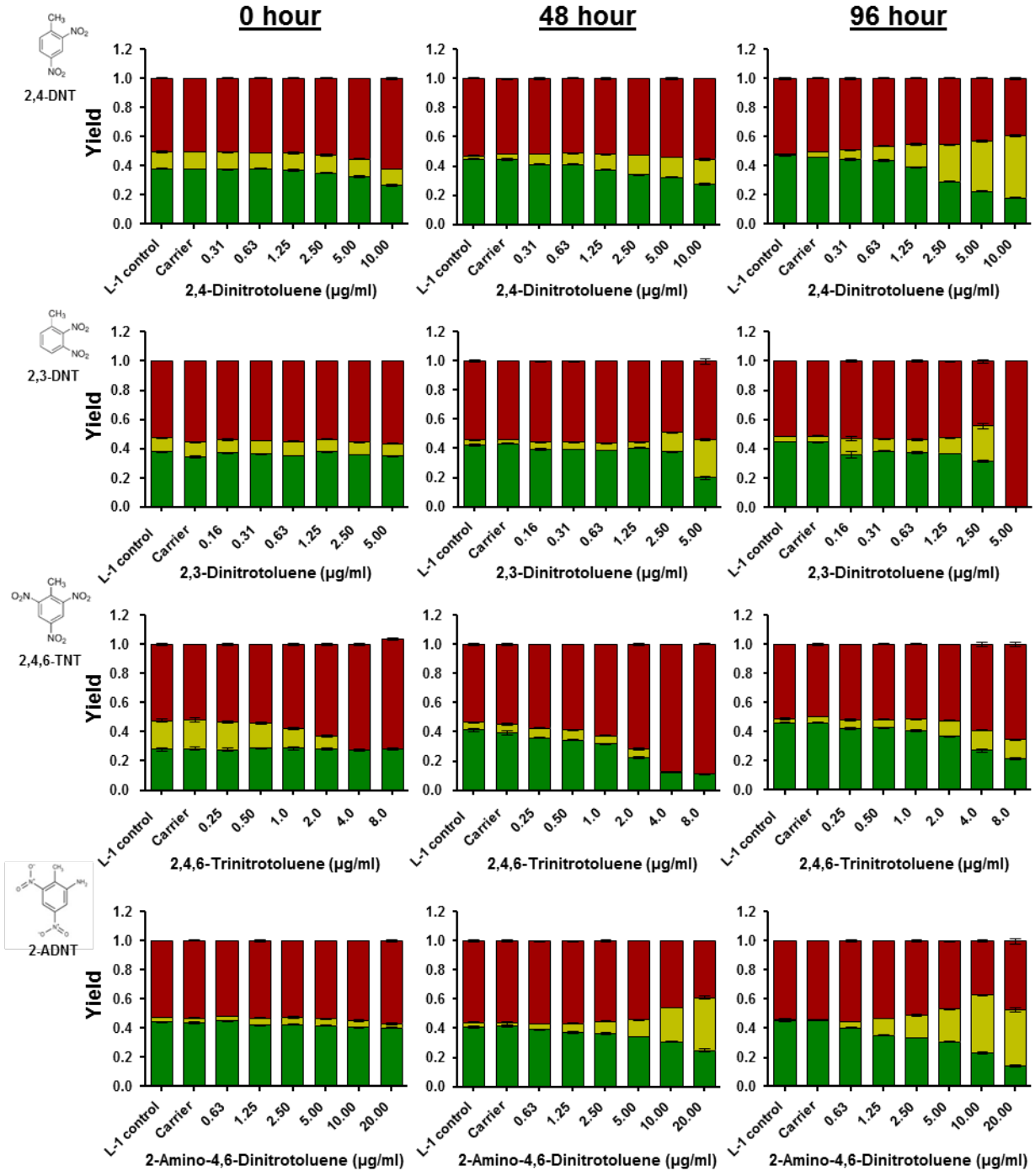
Flot, J.-F., H. Magalon, C. Cruaud, A. Couloux, and S. Tillier. 2008. Patterns of genetic structure among Hawaiian corals of the genus *Pocillopora* yield clusters of individuals that are compatible with morphology. C. R. Biologies. **331**:239-247.

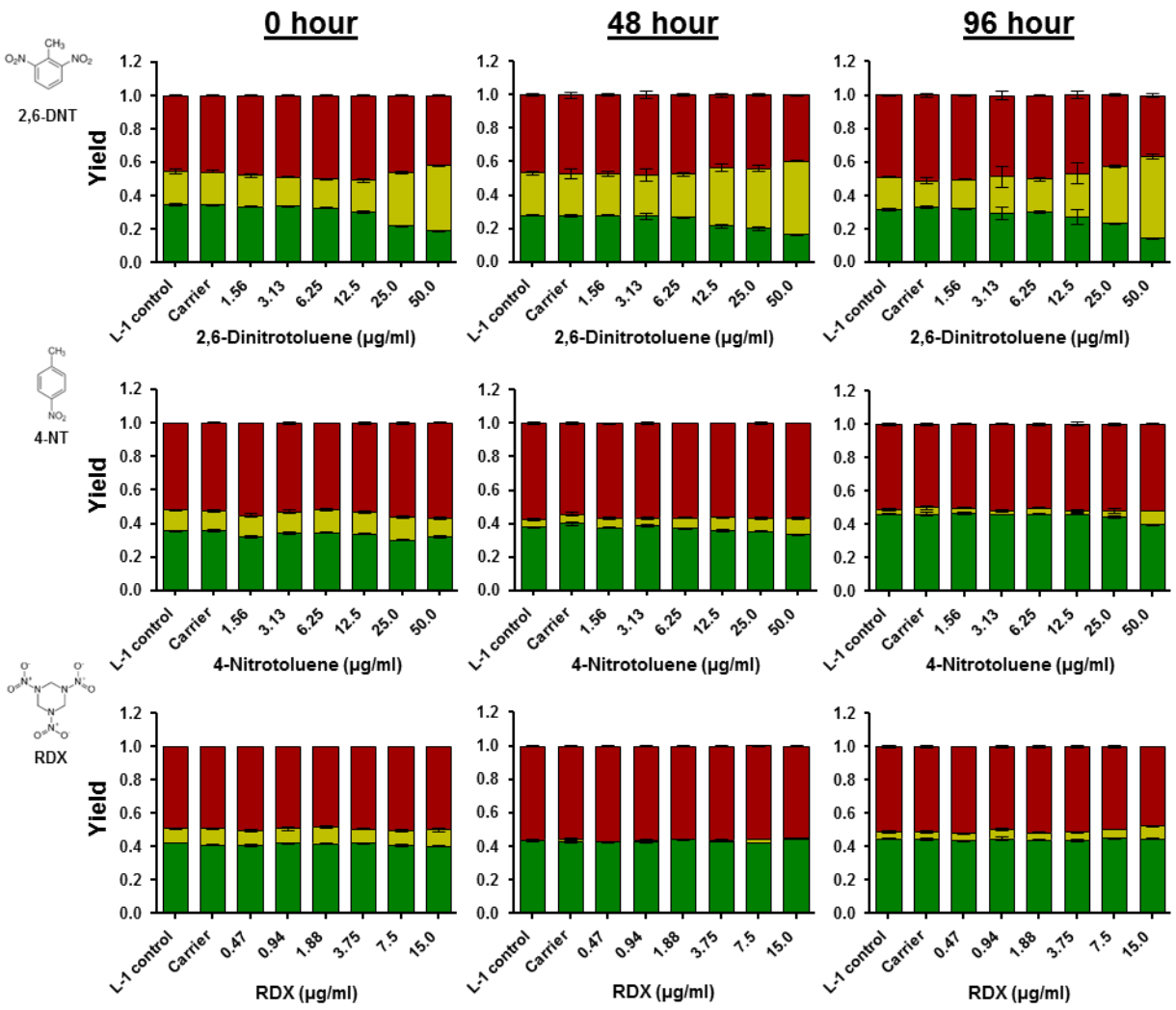
Hunter, S. E., D. Jung, R. T. Di Giulio, and J. N. Meyer. 2010. The QPCR assay for analysis of mitochondrial DNA damage, repair, and relative copy number. Methods. **51**:444-451.

Qiu, P., H. Shandilya, J. M. D'Alessio, K. O'Connor, J. Durocher, and G. F. Gerard. 2004. Mutation detection using Surveyor® nuclease. Biotechniques. **36**:702-707.

Appendix IV

Kautsky light curves of *Symbiodinium* cultures exposed to munitions compounds.




0 hour
48 hour
96 hour
2,6-DNT
4-NT
RDX
Yield
1.2
1.0
0.8
0.6
0.4
0.2
0.0
L-1 control
Carrier
1.56
3.13
6.25
12.5
25.0
50.0
2,6-Dinitrotoluene (μg/ml)
4-Nitrotoluene (μg/ml)
0.47
0.94
1.88
3.75
7.5
15.0
RDX (μg/ml)